Effect of Malnutrition in Infancy on the Development of Bone, Muscle and Fat[1]

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Measurements of the growth of African children show a number of differences from European averages. Data collected at child welfare clinics in Uganda (Welbourn, '55a) show that, despite low birth weights, in the average weighing about 6¾ pounds, weight gain in the first month often overtakes the European average, and exceeds it until the fifth or sixth month. At this point, weights fall below the European average, and may not approach it again until the third year. This retardation is associated primarily with a change to a starchy diet, either as a supplement or at the time of weaning, and is less marked in tribes whose staple foods have a higher protein content (Welbourn, '55b). Similar patterns of weight gain in childhood have been reported from other tropical regions (cf. Malcolm, '52; Millis, '54; Scragg, '55).

In later childhood and adolescence, the weights of children in the tropics are commonly below those of European children (cf. Grant, '51). In Uganda, McFie ('56) observed that average weights and heights of children from different regions tended to follow the general course of European averages (e.g., showing an increased upward trend at puberty) but were uniformly lower. As there was some uncertainty about the exact ages of the children, their measurements were also examined by plotting weight against height for each child on a logarithmic scale (Wetzel, '41). The distribution of points (fig. 1) showed that the general trend of growth followed that of European children (i.e., ran parallel to Wetzel's "middle channel") but that the majority lay on the "height" side of the line, indicating that the children tended to be light for their height. It was demonstrated that differences in height/weight proportions were associated with the nutritional status of the regions from which the children came, children from better-nourished areas coming nearer to European proportions. That these differences are not limited to non-European children is made clear by the similar observations reported for white children in Alabama by Dreizen et al. ('53).

From the relative difference between heights and weights, it appeared that at least one aspect of growth, that of bone length, was less influenced by the prevailing nutritional differences; clearly, the difference from well-nourished children must be associated with some other aspect of growth. The tissue reduction most likely to account for these changes is that of fat, which shows considerable variation with nutrient intake (McCance and Widdowson, '51; Keys and Brozek, '53). In severe cases of malnutrition, there is evidence that bone growth is retarded (Jones and Dean, '56), and there is a possibility that in the groups under consideration a lesser degree of malnutrition might have resulted in decreased bone width or density. The growth of nervous tissue and its integuments, on the other hand, seems less likely to be affected; despite low birth weights, head circumferences of Uganda babies were not below the European average (Scotland, '56); and Akim et al., ('56) noted no difference in psychomotor development between children who had shown diminished weight gain during infancy and those with normal weight gain.

There is, however, some evidence that differences between groups of different nutritional background may be associated

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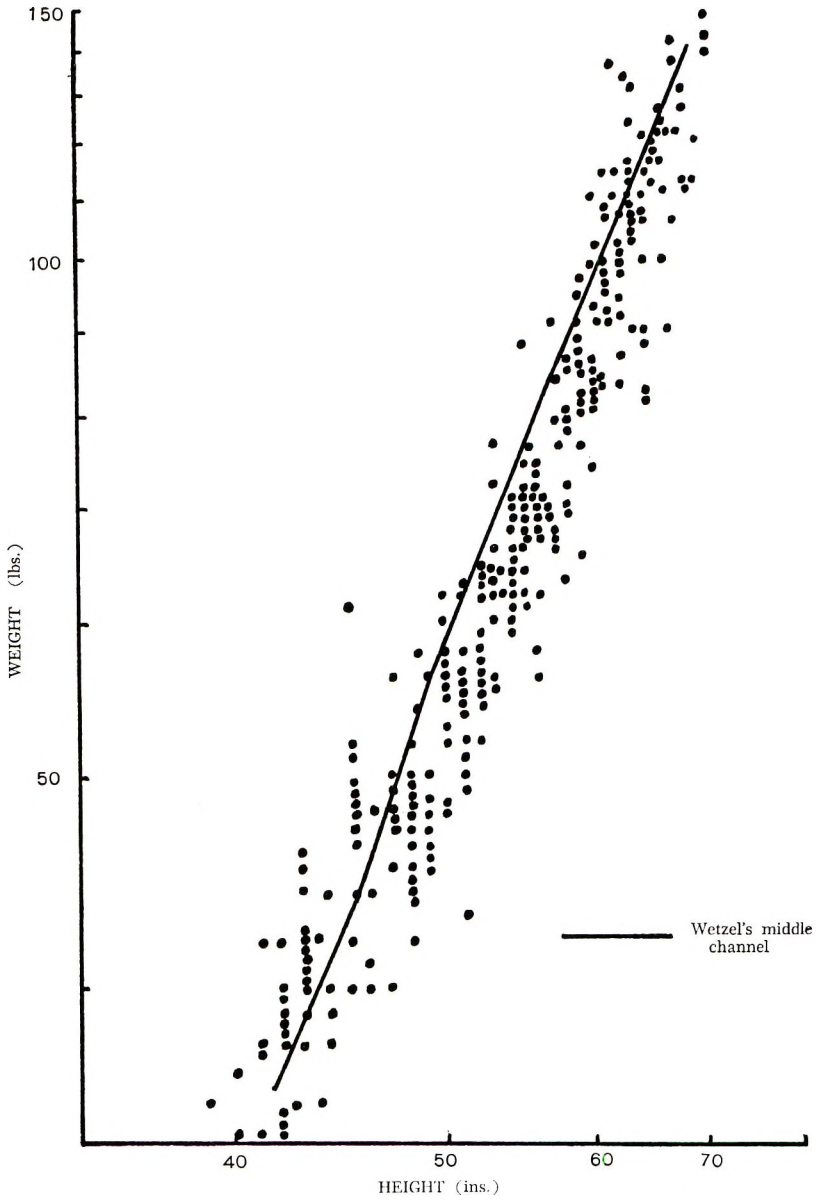


Fig. 1 Weight-height measurements of 289 African children, from 6 villages (McFie, '56). The scales on both axes are logarithmic.

with differences in bulk of muscle. Precise measurements were made by Stuart and co-workers of radiographs of soft tissues of the legs of children in Boston (U.S.A.), Marseille, and Madrid (Stuart and Kuhlmann, '42; Robinson et al., '42). Particularly in the Madrid children there was evidence of undernutrition from an early age; and the authors found that their weights were low in relation to their heights in comparison with other groups (cf. the Uganda children), and also that not only were their calf breadths less, but "smaller measurements for the muscle appear to be a more important and consistent factor in affecting total calf breadth than

those for skin and subcutaneous tissue." Measurements of African subjects, though less direct, suggest similar conclusions. Orr and Gilks ('31) compared children of the better-nourished Masai people with the Kikuyu, finding a similar superiority of weight-for-height in the former, and also observed that "from the earliest age the Masai give evidence of superior strength. At the maximum the males achieve an average (dynamometer) hand pressure of 88.1 pounds, which is 30 pounds more than the maximum for Kikuyu males." Similar results were reported in a study of three groups of males in Ruanda-Urundi (Hiernaux, '52): (a) Bakiga, well-nourished since childhood, (b) Bahutu, poorly-nourished, and (c) Bahutu of the same origins as (b) but working in the mines and receiving a highly nutritive diet. Only in the first group were the weights, girths of arm and calf, and dynamometer grips significantly greater than in the others, and Hiernaux concluded that the advantage of better nutrition in childhood outweighed the effects of good diet and exercise in the adult. Malcolm ('52, '54, '56) measured calf circumferences of young children on various islands in the South Pacific, and her data show that the typical "weaning" retardation in weight gain is associated with considerable and prolonged reduction in calf girth.

There is, then, considerable evidence that the consistently observed differences in height/weight proportions, between groups in tropical regions and European norms, are associated with different degrees of development of individual tissues. Furthermore, there is evidence that the differences in development are related to differences in nutritional background, and particularly to different levels of nutrition during early childhood.

EXPERIMENTAL PROCEDURE

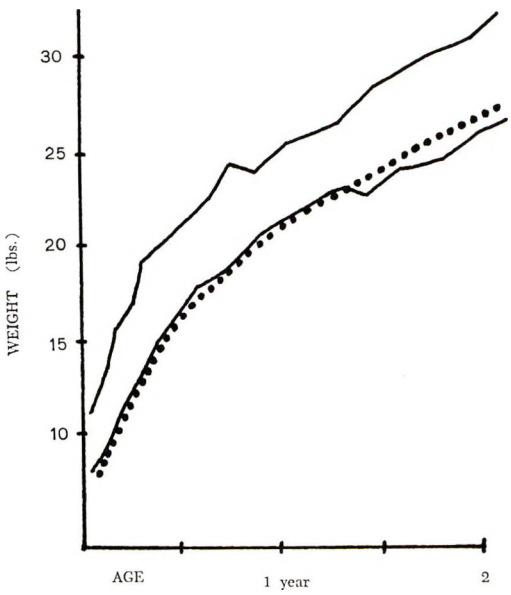
In order to investigate the relationship between the growth of tissues and the adequacy of nutrition during infancy, we have endeavored to use the radiographic technique of Stuart and co-workers on groups of children reared on different "planes of nutrition." The children were selected from the records of the Child Welfare Unit (Welbourn, '56), on the basis of regular attendance during the first two years of life, who were not less than three years and 6 months and not more than 7 years 6 months at the time of the study, whose whereabouts could be traced, and who could be brought to the hospital for radiography: in all, 52 children were available.

The children were grouped as follows by comparing their weight records with English averages (Tanner, '58): (a) *high*: those whose weights did not fall below the standard during the first 18 months; (b) *low*: those whose weights did not rise above the standard during the first 18 months; and (c) *falling*: those whose weights were at some time in their first year above the standard, but had by the end of that year fallen below the standard. The range of growth curves in each group is shown in figure 2 a, b and c.

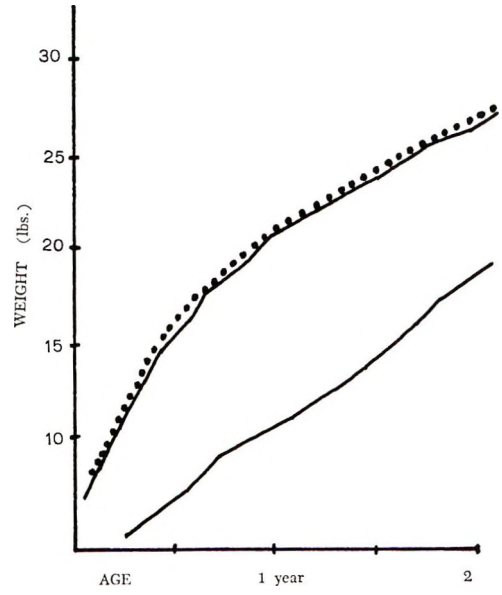
1. Radiographic measurements.[4] X-ray pictures were taken of the middle part of the right calf, with a tube–film distance of 36 inches. The child was lying on its back, the film was placed under the calf, and the heel was supported on a sandbag so that the posterior aspect of the calf just touched the upper surface of the film. From these pictures measurements were made at the level of greatest calf width of (a) the width of the fibula ("bone width"); (b) the total thickness of skin and subcutaneous fat on each side: as the contribution of skin to this measurement is assumed to be constant (approximately 1.5 mm on each side) the measurement is referred to as "fat"; and (c) the total width of muscle, by subtraction of the widths of tibia, fibula, and skin and subcutaneous fat from the total calf width. Examples of the radiographs are shown in figure 3.

The means of these measurements in each group are shown in table 1. The significance of the variations of the means between groups has been examined by analysis of variance using Yates' method for unequal subgroup numbers, also taking into account variations in age by subdividing the groups into those below 5 years, those from 5 years to 5 years

[4] We are indebted to Dr. A. G. M. Davies (Radiologist, Mulago Hospital, Kampala) for sparing x-ray film for this study.



(a)



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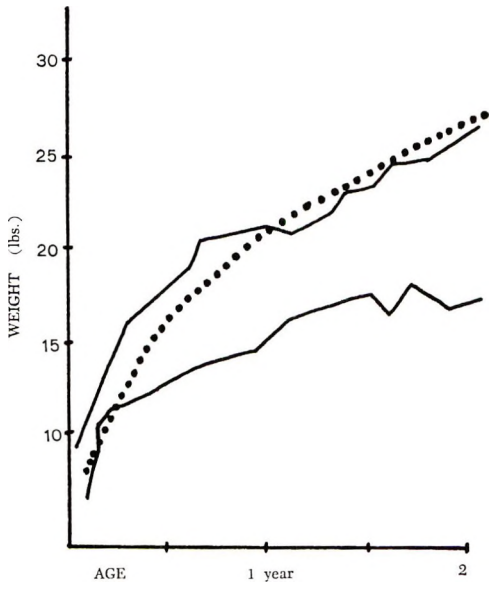

(c)

Fig. 2 Range of weight measurements in each group. The dotted line represents the English average, and the continuous lines the upper and lower limits in each group; (a) indicates "high" group; (b), "low" group; and (c), "falling" group.

11 months, and those of 6 years and above. The results of the analyses are shown in table 2; the variations associated with the type of growth are significant for muscle and for bone width, but not for fat.

These analyses do not, however, establish the significance of differences between any pair of means. These have been examined by calculation of the values of t (table 3). For the measurements of muscle and of bone width, the most significant differences are between the means of the "high" and the "falling" groups.

2. *Surface measurements.* In addition to the above, surface measurements were made of the length of the right humerus, from acromion to olecranon, with the forearm held in 90° flexion; of the girth of the right arm (flexed) at the midpoint of the above measurement; and of the triceps skin-fold, using the Harpenden calipers (Edwards et al., '55). It has been suggested by one of us (McFie, '56) that an estimate of the diameter of muscle might be calculated from these measurements by dividing the girth by π and subtracting the skin-fold thickness. This estimate (fig. 4) will be slightly overcorrected for fat, as the triceps fold is usually the largest skin-fold on the upper arm, and the estimate

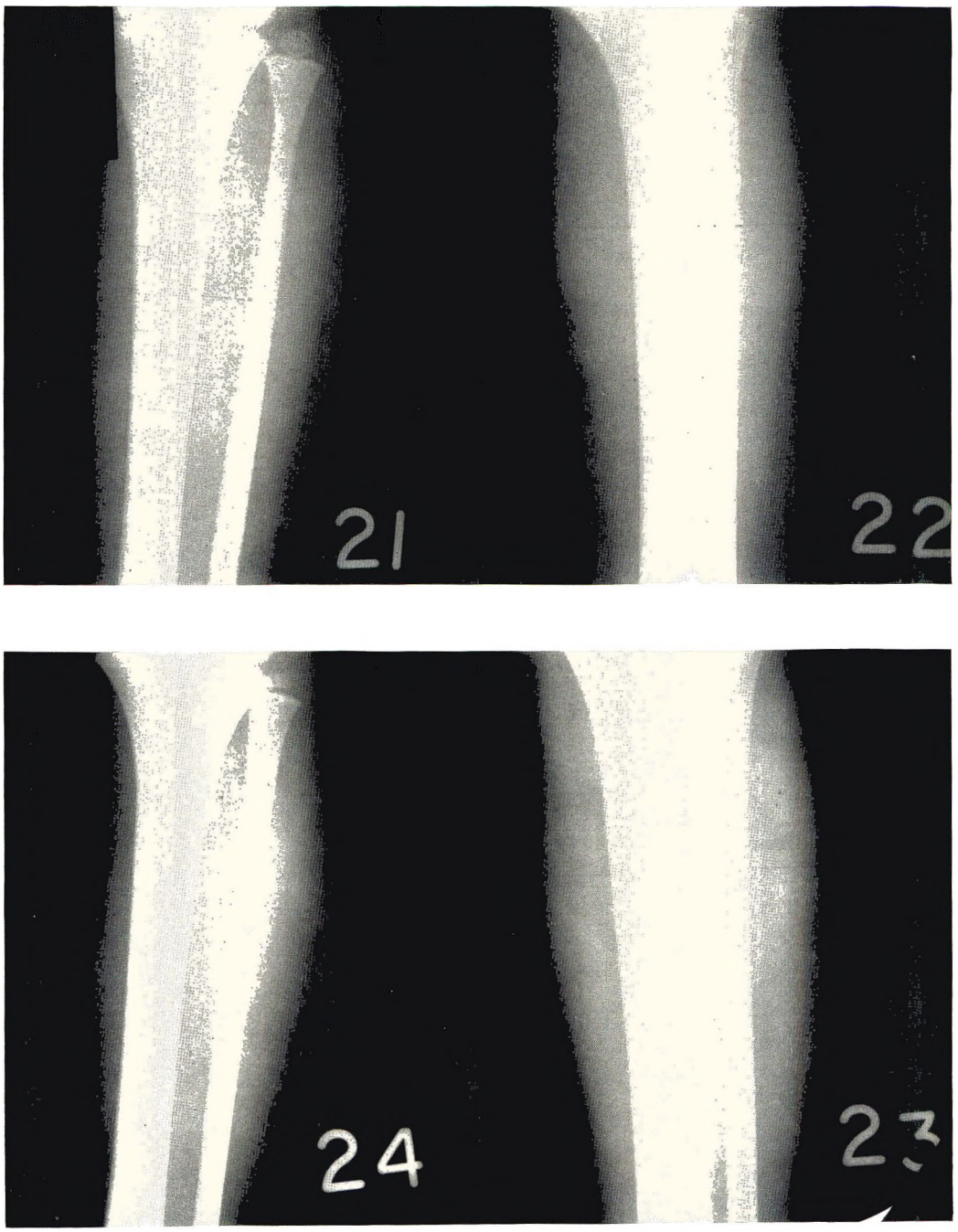

Fig. 3 Example of radiographs from which measurements were taken.

TABLE 1

Mean radiographic measurements of groups

	"High"	"Low"	"Falling"
No. in group	14	18	20
(a) Bone width, mm	7.4	6.6	6.5
(b) Subcutaneous fat, mm	10.1	9.1	9.4
(c) Muscle, mm	36.9	34.7	33.3

includes the diameter of the humerus; this is however proportionately much less than that of the muscle.

The validity of this estimate has been examined by correlating the estimate for each child with the radiographic measurement of muscle: the resulting correlation coefficient $r = 0.55$. Correlation of the triceps skin-fold with the radiographic measurement of skin and subcutaneous fat is $r = 0.66$. Both these correlations are significant at the 0.1% level. The means of the humerus length, muscle estimate, and skin-fold measurements for each of the three groups of children are given in table 4. Analysis of variance shows that only the variations in the estimate of muscle are statistically significant (table 5).

DISCUSSION

The evidence presented indicates that between three groups of children (a) whose weights had been maintained at a high level from birth, (b) whose weights had been at a consistently low level, and (c) whose weights had started at a high level but had, during the first year, fallen below this, there were differences in the

TABLE 3

Significance of differences (values of t) between group means

	"High"	"Low"
Bone width		
"Low"	3.00*[1]	—
"Falling"	3.38**	0.36
Muscle		
"Low"	2.06*	—
"Falling"	3.45**	1.44

[1] Significance of t: * = $P < 0.05$; ** = $P < 0.01$.

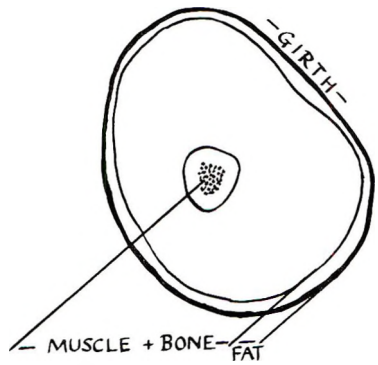

Fig. 4 Transverse section at midpoint of upper arm, to show relationship of surface measurements.

TABLE 2

Analyses of variance of radiographic measurements[1]

Source of variance	Sums of squares	Degrees of freedom	Mean squares	Variance ratio (F)
(a) Bone width				
Growth	693.94	2	346.97	5.69**[2]
Age	222.81	2	111.41	1.83
Residual	2865.55	47	60.97	
Total	3872.20	51		
(b) Subcutaneous fat				
Growth	299.14	2	149.57	0.34
Age	277.47	2	138.74	0.31
Residual	20866.39	47	443.97	
Total	21444.00	51		
(c) Muscle				
Growth	9241.33	2	4620.67	5.15**
Age	21603.04	2	10801.50	12.04***
Residual	42160.30	47	897.03	
Total	73004.67	51		

[1] In each analysis, growth × age interaction was found to be insignificant and its sum of squares was pooled with the residual.
[2] Significance of F: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

TABLE 4

Mean surface measurements of groups

	"High"	"Low"	"Falling"
Humerus length, cm	21.1	21.5	20.5
Muscle estimate, cm	4.79	4.58	4.46
Triceps fold, mm	8.1	7.6	7.6

measurements of different tissues made later in childhood.

In interpreting these differences, the first question must be whether the association between rate of weight gain in infancy and measurements of tissues is genetically determined or is due to differences in nutrition. Evidence has been presented (Hewitt, '58) of close correlations between heights, weights and tissue measurements in pairs of sibs, indicating a measure of genetic control of rates of growth. In contrast with African children, however, the children in these studies were for the most part well nourished, so that differences in nutrition were almost eliminated as a source of variation. In fact, in the older children, when there was more of a likelihood of diets diverging, there was a decline in the correlation for fat measurement. According to Tanner,[5] patterns of weight change corresponding to our "falling" group are rarely found in contemporary longitudinal studies in England. In our Uganda children, on the other hand, the "falling" group is the largest single group, and there is sufficient evidence of variation in diet in the first year of life to suggest that this is the main factor in determining the changes in weight gain.

The measurements of each tissue may be considered separately. The absence of significant differences in *bone length* between the groups is consistent with the earlier observations that height (of which bone length is a major function) varies less with variations in nutritional background during childhood than does weight.

That there is no significant difference between measurements of *subcutaneous fat*, either radiographically or by skin-fold calipers, may at first seem surprising in view of the assertion that the differences in weight gain of the groups of children were due mainly to differences in nutrition. Our selection of groups, however, was based on evidence of the adequacy of nutrition during infancy; at the ages at which the children were measured, they would all have been taking their own food, rather than being dependent on what they were fed, and it is unlikely that there would have been similar group differences in nutrition. The evidence of similarity of subcutaneous fat measurements supports this view, and makes it unlikely that differences in measurements of other tissues were associated with differences in nutritional status at the time of measurement.

Significant differences between the groups in *bone width* emphasize the difference of this aspect of bone growth from growth in length. Variations in this measurement are consistent with the observation that differences in weight may be associated with differences in nutritional background; and our data suggest that reduction in this aspect of growth is associated with reduced nutrition after the first months of life.

The significant differences between the groups in measurements of *muscle*, either radiographically or by inference from surface measurements, are in accord with the evidence of earlier studies, that differences in the development of muscle are associ-

[5] Personal communication.

TABLE 5

Analysis of variance of muscle estimate

Source of variance	Sums of squares	Degrees of freedom	Mean squares	Variance ratio (F)
Growth	77.26	2	38.63	3.78*[1]
Age	241.56	2	120.78	11.82***
Residual	480.52	47	10.22	
Total	799.34	51		

[1] The significance of F is indicated as in table 2.

ated with differences in nutritional background. Our technique of measurement approximated closely that of Stuart and co-workers with European children, and our results are in close agreement; they are also in agreement with the evidence relating to muscle development derived from studies of children in Africa and other tropical regions. As with bone width, our measurements of muscle show (tables 1, 3) that the major differences are between the "high" and "falling" groups, indicating that reduction in this aspect of growth is related to reduced nutrition occurring after the first few months of life.

Since a genetic explanation of this selective association between weight gain in infancy and muscle and bone development appears to be unlikely, other mechanisms must be considered. It is possible that nutritional deprivation in early childhood might result in reduction of muscular activity in the individual; and this might in turn lead to reduced development of muscle and possibly of bone thickness. Although there is no direct evidence that the milder degrees of malnutrition, associated particularly with reduced protein intake, to which our subjects were exposed, have any general effect on activity, there is the evidence (Akim et al., '56) that psychomotor development of children between two and three years of age is not thus affected. Since developmental measurements at these ages are largely in terms of locomotor functions, this evidence would not support the foregoing hypothesis.

An alternative explanation may be that during their development different tissues are particularly susceptible to the effects of reduced nutrition at different stages in the life of the individual. Such an explanation would be afforded by the "heterogonic" theory of growth of Hammond ('44) and co-workers. They have demonstrated in a wide variety of animals that during growth each tissue has a period of maximal development, and that these periods follow each other in a succession determined by their relative metabolic rates. This succession is, in the fetus: nervous system, viscera, bone (growth in length preceding growth in width); and immediately after birth: muscle, followed by fat. Furthermore, the extent of development of each tissue in the adult is affected by the "plane of nutrition" during which its maximal development took place: "during fetal life to maturity any part, organ or tissue of the animal body is proportionately most retarded in development by restricted nutrition at the age when it has its highest natural growth intensity" (Pálsson, '55). The weight-gain curves of our subjects (fig. 2 a, b, c) correspond qualitatively to those of animals in Hammond's experiments: "high" to "high-high," "low" to "low-low," and "falling" to "high-low." (We found no children who corresponded to Hammond's "low-high" group, i.e., who were below the standard in their first year but rose above it later.) In the terms of the experiments, this would identify the "critical" period for the development of the affected tissues as after the first stage; i.e., after the first months of life.

Our data are consistent with the view that the same succession in the development of tissues as occurs in animals, occurs also in man with the one modification, that birth in man occurs slightly earlier in the sequence, during rather than after the development of bone. On this hypothesis the period of "highest natural growth intensity" of muscle would occur not immediately after birth, but a few months later — the period during which nutritional deficiency, particularly of protein, is most marked in tropical diets. This would explain reductions in calf girth during this period (Malcolm, '54, '56); and the lasting effect of malnutrition at this age on the development of muscle would account for the failure of a nutritious diet and exercise in later years to improve limb girths and dynamometer grip (Hiernaux, '52). On the same hypothesis, the only effect of a high level of nutrition after this critical period would be an increase in the amount of body fat.

SUMMARY

Falling-off in weight gain during the first year of life is frequently noted in children in tropical regions, and is associated with inadequate protein intake. From welfare clinic records, a group of children has been selected who showed this falling curve of growth, and others who did not.

Soft tissue radiographs of the legs and surface measurements of the arms of these groups showed that the measurements of bone width and of muscle of the "falling" group were significantly different from those of other groups. Bone length and subcutaneous fat measurements were not significantly different.

It is suggested that this selective effect is not genetically determined, nor the consequence of limitation of activity, but that it is consistent with the application to man of Hammond's "heterogonic" theory of growth.

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Protein Reserves: Relationship of Dietary Essential and Nonessential Amino Acids to Formation and Maintenance in the Fowl[1]

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The alterations in body proteins that accompany protein depletion or repletion are frequently explained by the concept of protein reserves. The existence of protein reserve has been emphasized by Whipple ('48), Allison ('55) and others. But, the exact nature of these reserves is still in doubt (Hruza, '56). Allison and Wannemacher ('57) consider the protein reserve to be the tissue proteins that rise and fall with corresponding increases and decreases in protein intake. Plasma albumin and the cytoplasm of the liver and gut have been considered to be such proteins, because they are very sensitive to dietary protein change (Addis et al., '40; Kosterlitz, '47; Whipple, '48; Allison, '55).

Support for the concept of protein reserve in the laying hen has ben presented by Leveille et al. ('61). Evidence has also been provided for the existence of protein reserve in the adult rooster by Leveille and Fisher ('58). These workers maintained mature roosters in nitrogen equilibrium after they reached an endogenous plateau of nitrogen excretion (using a N-free diet) by feeding an amino acid mixture simulating whole-egg protein at one-half the determined nitrogen requirement. Subsequent determination of the amino acid requirements of the rooster (Leveille et al., '60) revealed that this amino acid mixture, although it provided only one-half the nitrogen requirement, did nevertheless satisfy the essential amino acid requirements. Thus the specificity of the reduced dietary nitrogen requirement remained unanswered. It was, therefore, the purpose of the investigations reported herein to determine the nature of this dietary nitrogen and, consequently, the relationship of the essential and nonessential amino acid portions of the nitrogen requirement to the protein reserve status of the adult rooster.

EXPERIMENTAL

Mature White Leghorn roosters selected on the basis of body weight were maintained in individual metabolism cages in a temperature-controlled room (65 to 72°F.) throughout the experiments. They were fed for a 10-day standardization period either a nitrogen-free or a purified diet[2] that contained a sufficient quantity of whole-egg protein to satisfy the nitrogen and amino acid requirements for maintenance. Following the standardization period the roosters received test diets for 7-day periods. Two 48-hour collections for nitrogen determination were carried out during the last 4 days of each test phase.

The mixed excreta (urine and feces) were homogenized with water and diluted to 500 or 1000 ml. An appropriate aliquot was taken for nitrogen analysis by the semimicro Kjeldahl method. In two experiments samples were digested by the Kjeldahl method, and nitrogen was determined with an auto-analyzer by modification of the sodium phenate, hypochlorite colorimetric method (Ferrari, '60).

In the final series of experiments blood samples were taken from a wing vein for the determination of hematocrit, plasma protein and amino nitrogen. Plasma protein was determined with an auto-analyzer using the biuret color reaction. Amino nitrogen was determined by the method of

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[2] Some of the vitamins were generously supplied by Merck Sharp and Dohme, Rahway, New Jersey.

Danielson as presented by Hawk et al. ('54).

The composition of the standardization diets and all test diets is shown in table 1. The whole-egg standardization diet was designed to provide 280 mg of N per kg body weight per day and 95 Cal. of metabolizable energy per kg per day.[3] The nitrogen of all tests diets was provided by amino acids. In all cases, 10% of the essential amino acid N was provided by unavailable D-isomers (Leveille and Fisher, '60). Test diets 1 and 2 furnished 190 mg of N per kg per day and 95 Cal. of metabolizable energy per kg per day. Test diet 1 provided the maintenance requirement for essential amino acids (Leveille et al., '60), which contributed 115 mg of N per kg per day. Test diet 2 contained approximately two-thirds the essential amino acid maintenance requirement (75 mg N per kg per day) and proportionately more glycine and glutamic acid to provide also 190 mg of N per kg per day. Test diets 3 and 4 contained, respectively, 300 and 245 mg of N per kg per day. Test diets 5 and 6 supplied 152 and 350 mg of N per kg per day. Diets 3 through 6 satisfied the essential amino acid (115 mg of N per kg per day) and energy (95 Cal. per kg per day) requirements of the rooster. The actual N intake for the various diets generally agreed closely with the calculated value except for a few instances where the animals reduced their food consumption.

RESULTS

The first series of experiments was designed to determine whether the decreased nitrogen (N) requirement between animals in full protein reserve and those depleted of their reserve (endogenous state) is due

[3] Calculated from the metabolizable energy values of Anderson ('55).

TABLE 1

Composition of basal ration and test diets

Basal ration		Essential amino acid mixture[1]		
	%		*% of diet*	*mg/kg/day*
Glucose	15.00	L-arginine·HCl	0.56	145
Corn oil	12.00	L-lysine·HCl	0.14	36
Dextrin	5.00	L-leucine	0.48	124
Fiber	4.00	DL-isoleucine	0.56	145
Minerals[2]	3.00	DL-valine	0.23	61
Antacid adsorbent[3]	1.00	DL-threonine	0.56	145
Choline chloride	0.24	L-tryptophan	0.07	19
Vitamin A, D and E mix[4]	0.15	DL-methionine	0.27	71
B-vitamin mix[4]	0.15	L-phenylalanine	0.10	26
Corn starch	to 100	L-cystine	0.16	42
		L-tyrosine	0.13	31

Nitrogen portion of standardization and test diets

Diet	Whole egg[5]	Essential amino acid mixture	Nonessential amino acids	
			Glutamic	Glycine
	%	%	%	%
Standardization				
Whole egg (280 mg N/kg/day)	16.00	—	—	—
N-free	—	—	—	—
Test				
1 (190 mg N/kg/day)		3.26	1.09	0.58
2 (190 mg N/kg/day)		1.98	1.99	1.05
3 (300 mg N/kg/day)		3.26	3.28	1.73
4 (245 mg N/kg/day)		3.26	2.46	1.29
5 (152 mg N/kg/day)		3.26	0.67	0.36
6 (352 mg N/kg/day)		3.26	4.89	2.50

[1] Calculated on basis of feeding 26 gm/kg/day.
[2] For composition see Fisher et al. ('60).
[3] Gelusil, Warner Chilcott, Morris Plains, N. J.
[4] For composition see Fisher and Johnson ('56).
[5] Whole egg contained 44.12% protein.

to a simultaneous or independent decrease in the need for essential or nonessential amino acids, or both.

The nitrogen excretion, and, consequently, the protein reserve status of 20 roosters were standardized at the endogenous level by feeding a N-free diet to all animals for 10 days. Leveille and Fisher ('58) have shown that the rooster reaches an endogenous N excretion level after receiving a N-free diet for 4 days. In the present study the N excretion also began to reach a plateau after 4 days. The first bar on the left side of figures 1 and 2 shows the N excretion obtained during the last two days the N-free diet was fed; this was considered to be the endogenous excretion level representing depletion of protein reserve. The average excretion of 135 mg of N per kg per day is in excellent agreement with the endogenous level noted by Leveille and Fisher ('58).

After the endogenous excretion level was reached, the 20 roosters were divided into two groups of 10 each. One group was fed test diet 1 (115 mg of N per kg per day of essential and 75 mg of N per kg per day of nonessential amino acids) and the other received test diet 2 (75 mg of N per kg per day of essential and 115 mg of N per kg per day of nonessential amino acids). Diets were reversed after a 7-day feeding period;

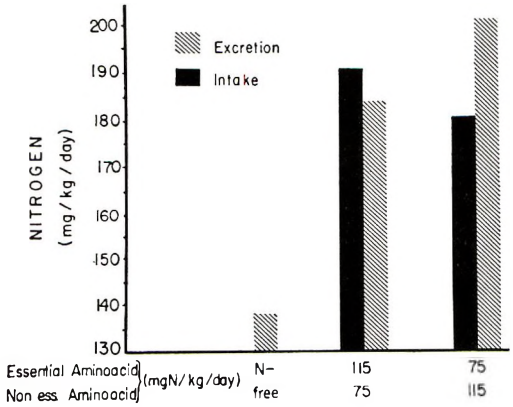

Fig. 1 Nitrogen balance studies with 10 adult roosters depleted of their protein reserves and fed approximately two-thirds (190 mg N per kg per day) of their nitrogen requirement for maintenance. These results were obtained from roosters started with test diet 1 and changed to test diet 2. The intake bar represents actual N consumption, whereas the values underneath the abscissa are the calculated N intake.

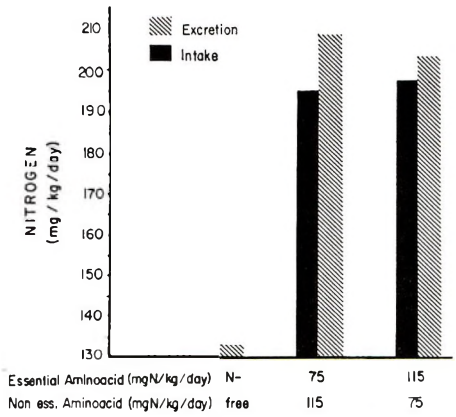

Fig. 2 Nitrogen balance studies with 10 adult roosters depleted of their protein reserves and fed approximately two-thirds (190 mg of N per kg per day) of their nitrogen requirement for maintenance. These results were obtained from roosters started with test diet 2 and changed to test diet 1. The intake bar represents actual N consumption, whereas the values underneath the abscissa are the calculated N intake.

birds formerly receiving test diet 1 received diet 2 and those fed diet 2 then received test diet 1.

In figures 1 and 2 it is clearly illustrated that roosters consuming quantities of nitrogen below the maintenance requirement can achieve N equilibrium provided the essential amino acids are furnished at the maintenance requirement level (test diet 1). If, however, as for test diet 2, the essential amino acids are reduced below the maintenance level, the N equilibrium could not be attained by increasing the nonessential amino acids to insure the same N intake as for test diet 1. Thus a significant decrease ($P < 0.01$) in N balance was observed when the diets of the roosters were reversed from test diet 1 to diet 2 (fig. 1) and a significant increase ($P < 0.05$) when they were changed from test diet 2 to test diet 1 (fig. 2). The results indicate therefore that the decreased N requirement for roosters depleted of their protein reserve is reflected *only* in a decreased requirement for nonessential amino acids.

The relationship of dietary nonessential amino acids to the protein reserve status of the rooster is further illustrated in figure 3. These data were obtained from 20 roosters fed a diet providing 300 mg of N per kg per day (test diet 3) designed to satisfy the es-

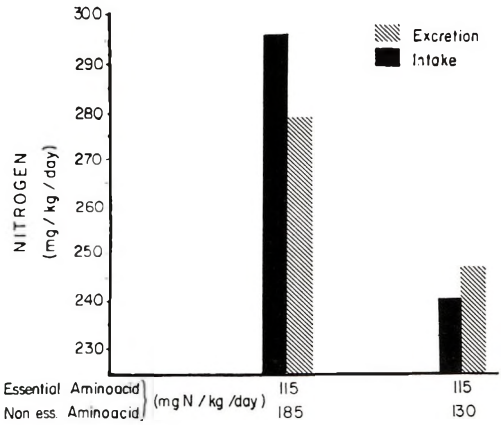

Fig. 3 Effect of lowering nonessential amino acid N on N balance of 20 nondepleted adult roosters. The intake bar represents actual N consumption, whereas the values underneath the abscissa are the calculated N intake.

sential amino acid maintenance requirements. After the roosters received this diet for a 7-day period, they were fed the same diet for another 7 days; but this was so formulated as to provide 245 mg of N per kg per day (test diet 4) by substituting glucose for 55 mg of N from nonessential amino acids. In figure 3 it is again shown that the rooster can maintain N equilibrium at a N intake below the maintenance requirement provided the essential amino acids are present in adequate amounts. More revealing, however, is the significant ($P < 0.01$) decrease in N balance when the roosters were changed from a diet designed to support full protein reserves (test diet 3, 300 mg of N per kg per day) to a diet suboptimal in N (test diet 4, 245 mg of N per kg per day). Thus these data also suggest that the protein reserves can be maintained by a dietary source of nonessential amino acids.

A final series of experiments was executed to determine whether the protein reserves of the adult rooster could be replenished by dietary nonessential amino acids. Ten roosters were fed the whole-egg standardization diet that furnished 280 mg of N per kg per day. This level of whole-egg protein provided an excess of the essential amino acids and satisfied the N requirement for maintenance. By feeding this diet for 10 days, it was presumed that the protein reserve status of all animals would be equalized at a high level. After this standardization period the roosters were fed a N-free diet until an endogenous excretion pattern was noted (that is, until the protein reserves had been depleted). The animals were next given a level of N equivalent to the endogenous excretion (152 mg of N per kg per day, of which 115 mg of N per kg per day was provided by essential amino acids). This was followed by a 5-day repletion period during which they were given 352 mg of N per kg per day with the essential amino acid portion still providing only 115 mg of N per kg per day.

The results obtained in this experiment are summarized in figure 4. The area under the curve (N excretion) between the whole-egg and the N-free feeding periods represents depletion of protein reserves. The maintenance of N equilibrium (as illustrated by an essentially unchanged excretion) when birds were fed the level of N corresponding to the endogenous excretion demonstrates the adequacy of this ration for birds depleted of their protein reserves. This experiment confirms the previous observations that birds can be maintained in N equilibrium when fed levels of N below the maintenance requirement if the essential amino acids are provided at the maintenance level. During the final phase of this experiment the area under the curve reflects the repletion of protein reserves and hence the adequacy of dietary nonessential amino acids in this regard. Nitrogen balance is, of course, positive during this repletion period but the retention decreases (excretion increases, fig. 4) as it should when the reserves become filled.

On the last day of each period in the final series of experiments blood samples were taken for plasma protein, hematocrit, and amino N determination. A significant ($P < 0.01$) decrease in plasma proteins concurrent with the onset of an endogenous N excretion pattern was noted (table 2). When the protein reserves were repleted with dietary nonessential amino acids the plasma proteins started to increase. Plasma amino N also increased significantly ($P < 0.01$) when the roosters were fed the repletion diet containing 352 mg of N per kg per day. This was the only change observed in this plasma component

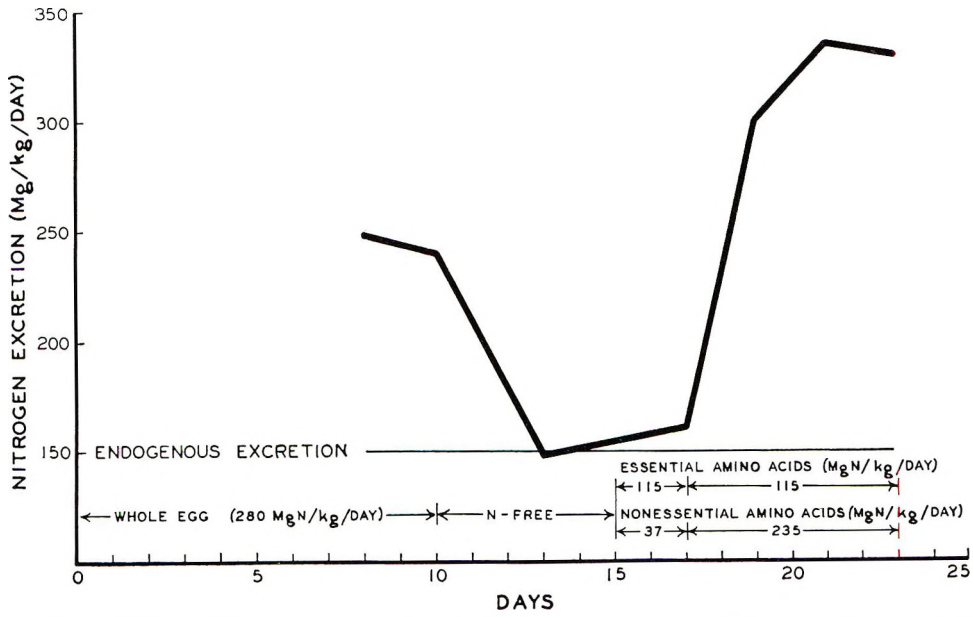

Fig. 4 Nitrogen balance studies with 10 adult roosters fed varying levels and sources of dietary N.

TABLE 2

Blood values of roosters depleted and repleted of their protein reserves

Period	Blood measurements[1]		
	Hematocrit	Plasma protein	Amino N
	%	*gm/100 ml*	*mg/100 ml*
Standardization[2]	44.3 ± 0.5	4.44 ± 0.09	8.31 ± 0.40
Depletion[3]	44.8 ± 0.6	3.78 ± 0.10	8.48 ± 0.20
Repletion[4]	43.4 ± 0.3	3.93 ± 0.10	9.87 ± 0.33

[1] Mean values with standard error from 10 roosters.
[2] Whole-egg diet, providing 280 mg of N/kg/day.
[3] N-free diet.
[4] Test diet 6, providing 352 mg of N/kg/day.

due to dietary treatment. The hematocrit readings in all instances did not change appreciably.

DISCUSSION

Although attempts have been made to clarify the composition and function of the protein reserves (Hruza, '56),[4] the nature of the dietary amino acids responsible for building or maintaining these reserves had not been elucidated. The results of the present experiments indicate that nonessential amino acids can maintain and replete the protein reserves without a simultaneous increase in the dietary essential amino acids. Thus, the decreased N requirement between birds in full and in depleted protein reserves was reflected only in a decreased nonessential amino acid requirement. But, a decrease in essential amino acid intake resulted in negative N balance. Again, during repletion of depleted birds, the increase in nonessential amino acids only, permitted the birds to go into strong positive N balance and rebuild their reserves.

That dietary nonessential amino acids can form and maintain protein reserves does not, of course, imply that reserve proteins exclusively contain nonessential amino acids. Rather it may be suggested that the labile nature of reserve proteins in conjunction with the dynamic state of certain body proteins permits metabolic shifts (namely, de- and transaminations)

[4] Halac, E., Jr. Reserve protein — a misnomer? Federation Proc., *19*: 326 (abstract).

to take place at a rate sufficient to provide those amino acids, peptides, or proteins necessary to maintain the protein reserves of the animal. Certain of the nonessential amino acids (namely, alanine, glutamic, and aspartic acid) play an important role in these intermediary metabolic reactions. It should not be overlooked, however, that a substantial fraction of the N increment considered to be the reserve protein may consist of nonprotein N both in the cells and in the extracellular fluid.[5]

The adequacy of nonessential amino acids for building and maintaining protein reserve suggests that proteins satisfying the essential amino acid needs within the total N requirement should be virtually of the same biological value. Support for this concept may be drawn from the data of Barnes et al. ('46), who showed that the biological value of egg protein for growing rats remained constant over a given range of protein absorption, despite a sharp decrease in utilization for maintenance purposes. This decreased utilization indicates an excess of amino acids which in the case of the growing rat (Barnes et al., '46) is used for growth, and which in an adult animal may be available for building or maintaining protein reserves. The utilization by the adult animal of excess amino acids in this manner may serve to explain the inability to produce an amino acid imbalance at the maintenance level (Fisher et al., '60), and supports Mitchell's ('59) concept of an aggregate amino acid requirement for growth and a particulate requirement for maintenance.

A difference was noted in dietary amino acids required to replete animals depleted primarily of their protein reserves (this study) and animals depleted for prolonged periods of time (Allison and Fitzpatrick, '60). In the latter case, after prolonged N depletion of adult animals, the amino acids necessary for repletion were observed to be similar to those necessary for growth. In the present study, in which only the protein reserves were depleted, nonessential amino acids sufficed. But this does not preclude the possibility that individual essential amino acid requirements are altered in the depleted animals.

Finally, the shifts in plasma protein observed in the last series of experiments provide corroborative evidence for changes in the protein reserve status of the adult rooster due to changes in dietary nonessential amino acids. The increasing plasma protein observed during repletion with 352 mg of N per kg per day reflects an increase in reserve protein although not to its maximal level. This is also indicated in figure 4 by the extent of N retention of birds repleted for 5 days, because a N excretion equivalent to the intake (352 mg of N per kg per day) would have been indicative of full reserves. The repletion of plasma protein and the constancy of the hematocrit values (a reflection of hemoglobin concentration) furnish additional support to our previous observations (Leveille et al., '60) of the nonessentiality of dietary histidine for the adult rooster.

SUMMARY

A series of nitrogen balance experiments was carried out to determine the relationship of the essential and nonessential amino acid portions of the nitrogen requirement to the protein reserve status of the adult rooster. It was observed that dietary nonessential amino acids are adequate in the formation and maintenance of protein reserves provided the maintenance requirement for essential amino acids is satisfied.

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Comparative Value of Dietary Rapeseed Oil, Sunflower Seed Oil, Soybean Oil and Animal Tallow for Chickens

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Stimulation of chick growth by supplementing rations with fat sources such as animal tallow, corn oil and soybean oil has been demonstrated (Pepper et al., '53; Aitken et al., '54; Rand et al., '58; Carew et al., '59). Little information is available concerning the influence of dietary rapeseed oil or sunflower seed oil on chick growth. Correspondingly, there is lack of information with respect to the influence these two fat sources may exert on metabolizable energy content of chick rations and on the composition of carcass adipose tissue.

The results reported herein compare the effects of dietary animal tallow, soybean oil, rapeseed oil and sunflower seed oil on chick performance, metabolizable energy content of the ration and fatty acid composition of chick carcass adipose tissue.

EXPERIMENTAL

Male broiler chicks obtained from a commercial hatchery were used in this study. At one day of age, 15 cockerels were randomly assigned to each experimental pen, wing-banded, group-weighed and placed in electrically heated brooder batteries with raised, wire-mesh floors. The cockerels remained in these pens until 4 weeks of age, at which time they were transferred to broiler finishing batteries. Each of the 9 ration treatments were assigned to the experimental pens according to a randomized block design. Weight gain and feed consumption data were recorded bi-weekly. Feed and water were supplied ad libitum.

The basal ration contained 3.1% of fat and 22.2% of protein. This ration contained the following ingredients expressed as percentage of the total ration: ground oat groats, 36.5; ground wheat, 38.0; soybean meal (45% protein), 13.0; meat meal (50% protein), 5.0; fish meal (70% protein), 2.5; dehydrated alfalfa meal (17% protein), 2.0; dicalcium phosphate, 1.5; vitamin premix, 1.0; salt premix, 0.5. The vitamin premix contributed the following per kilogram of ration: vitamin A, 7150 IU; vitamin D_3, 818 ICU; vitamin E, 22 IU; and the following expressed as milligrams per kilogram of ration; riboflavin, 5.5; niacin, 16.5; pantothenic acid, 11; choline, 275; menadione, 1.1; vitamin B_{12}, 0.01: The salt premix supplied the following, expressed as milligrams per kilogram of ration: sodium chloride, 4,600; manganese, 120; iron, 17; zinc, 11; copper, 6.6. Each fat source was substituted in the basal ration for an equivalent weight of wheat, and levels of wheat and soybean meal were adjusted to maintain a constant percentage of protein in the ration. Stabilized animal tallow, degummed soybean oil, degummed rapeseed oil and crude sunflower seed oil[1] were each tested at 4 and 8% of the ration.

The procedure described by Hill and Anderson ('58) was used for determining the metabolizable energy of the rations. This entailed the use of chromic oxide as an index substance in the rations (to establish the ratio of excreta produced per unit of feed intake), determining gross energy of feed and excreta in a Par adiabatic colorimeter, and computation of metabolizable energy values with correction being made for nitrogen retention. Nitrogen in feed and excreta was determined by the macro-Kjeldalh technique.

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[1] Soybean oil, rapeseed oil and sunflower seed oil kindly supplied by Co-operative Vegetable Oils Ltd., Altona, Manitoba.

To facilitate determination of metabolizable energy, total excrement was collected from each experimental pen for 5 consecutive days during the 4th and again during the 6th week of the experiment. Metabolizable energy of the rations was determined at the end of each 5-day collection interval as described previously.

The experiment was terminated when the cockerels were 8 weeks old. At this time, three randomly selected cockerels from each experimental pen were killed and the thyroid glands excised and weighed immediately. At the same time, approximately 15 gm of abdominal adipose tissue were removed from each cockerel. The samples of adipose tissue obtained from the three cockerels per experimental pen were pooled to form one composite sample. Fat was extracted from the composite samples according to the method described by the Association of Official Agricultural Chemists ('60). Quantitative analysis for fatty acid composition of the fat sources used in this investigation and adipose tissue was accomplished by gas chromatography. A Beckman GC-2 gas chromatograph was used and fatty acids (as methyl esters) were separated on a butanediol succinate polyester column with a flow rate of 190 ml per minute and a temperature of 200°C.

Data were subjected to analysis of variance (Snedecor, '56) and multiple comparisons made according to Duncan ('55).

RESULTS AND DISCUSSION

The data presented in table 1 show that weight gain and efficiency of feed utilization of chicks from zero to 8 weeks of age were improved by including various fat sources in the ration. In the case of weight gain, only the addition of 4% of animal tallow failed to result in a significant ($P < 0.05$) increase over the basal group. Efficiency of feed utilization was significantly ($P < 0.05$) improved by all fat additions except when 4% of animal tallow or 4% of rapeseed oil were used. The marked improvement in weight gain and feed efficiency due to adding soybean oil to the ration agrees with observations of Dam et al. ('59) and Carew et al. ('59). Aitken et al. ('54) reported a substantial stimulation of growth and increased efficiency of feed utilization of broilers when beef tallow was used at 10% of the ration. The present study indicates that 8% of dietary animal tallow stimulated growth and improved feed efficiency but that a level of 4% was relatively ineffective.

When used at 4% of the ration, rapeseed oil and sunflower seed oil were as effective in promoting weight gains as 4% of soybean oil and were more effective than 4% of animal tallow. All fat sources were similar in their growth stimulating properties when included in the ration at the 8% level. Rapeseed oil, which contains a large proportion of erucic (13-docosenoic) acid in its total fatty acid complement, was reported by Deuel et al. ('48), Thomasson ('55) and Beare et al. ('59b) to decrease body weight gain when fed to rats. Beare et al. ('59a) presented data showing that relatively high levels (5 to 11% of total ration) of ethyl erucate markedly retarded

TABLE 1

Comparative effects of dietary fats on weight gain, efficiency of feed utilization and thyroid gland weight of cockerels

Ration treatment	Av. weight gain 0–8 weeks	Feed/gain 0–8 weeks	Av. thyroid gland weight
	gm		*mg/kg body weight*
Basal (no added fat)	1396 ± 38[1]	2.14 ± 0.04	7.9 ± 1.3
Animal tallow, 4%	1412 ± 7	2.07 ± 0.04	7.4 ± 1.7
Animal tallow, 8%	1543 ± 25[2]	2.00 ± 0.02[2]	8.3 ± 1.5
Soybean oil, 4%	1485 ± 35[2]	2.00 ± 0.01[2]	9.7 ± 2.2
Soybean oil, 8%	1525 ± 28[2]	1.97 ± 0.02[2]	8.5 ± 1.4
Rapeseed oil, 4%	1499 ± 34[2]	2.06 ± 0.03	10.2 ± 1.7
Rapeseed oil, 8%	1513 ± 39[2]	1.94 ± 0.01[2]	7.7 ± 0.7
Sunflower seed oil, 4%	1560 ± 25[2]	1.97 ± 0.04[2]	12.0 ± 1.6
Sunflower seed oil, 8%	1477 ± 35[2]	2.00 ± 0.06[2]	7.1 ± 1.1

[1] Figures preceded by ± sign are standard errors of the mean.
[2] Difference between this mean and the basal group significant at $P < 0.05$.

growth of young rats and concluded that growth inhibition caused by high dietary levels of rapeseed oil was due to erucic acid. These workers also noted that depression of weight gain could be explained by reduced feed consumption of animals receiving a diet containing rapeseed oil or ethyl erucate.

The rapeseed oil used in the present investigation supplied about 2.5% of erucic acid to the total ration when the oil comprised 8% of the diet. There was no apparent harmful influence exerted by this amount of dietary erucic acid; in fact, weight gains were increased slightly.

Thyroid gland weights shown in table 1 indicate that little, if any, of the goitrogenic material normally found in whole rapeseed and rapeseed oil meal was present in the rapeseed oil tested. None of the fat sources studied caused any appreciable change in thyroid gland weight.

The data on metabolizable energy of the rations are shown in table 2. Values obtained for each ration during the 4th and 6th weeks of the experiment were pooled to arrive at the given data. This procedure appeared to be justified because the metabolizable energy values for each ration as determined during the 4th and 6th week were not significantly ($P < 0.05$) different.

When added to the ration at a 4% level, rapeseed oil and animal tallow were quite similar with respect to resulting metabolizable energy values. The ration containing 4% of sunflower seed oil had a metabolizable energy value slightly higher than the ration containing 4% of animal tallow and significantly ($P < 0.05$) higher than the ration with 4% of rapeseed oil. However, very close similarity between rapeseed oil, animal tallow and sunflower seed oil was observed when the fat sources comprised 8% of the ration, indicating that rapeseed oil and sunflower seed oil, as used in this investigation, are essentially equivalent to animal tallow in terms of resulting metabolizable energy. Deuel et al. ('48, '49) reported that rapeseed oil fed to the rat had a lower digestibility coefficient and slower rate of absorption than many other fat sources tested, whereas in humans the digestibility coefficient for rapeseed oil was about 99%. Beare et al. ('57) observed increased lipid excretion by rats receiving rations containing 10 to 20% of rapeseed oil, but this increase did not occur when lower levels (less than 8%) of dietary rapeseed oil were used. The comparative growth and ration metabolizable energy data presented herein indicate that energy supplied by up to 8% of dietary rapeseed oil was as available to the chick for subsequent utilization as energy derived from comparable levels of dietary animal tallow or sunflower seed oil.

Sibbald et al. ('61) reported that crude soybean oil possessed a considerably higher metabolizable energy value for chickens than animal tallow. In the present study, rations containing 4 to 8% of soybean oil had significantly higher metabolizable energy values than when corresponding levels of animal tallow, rapeseed oil or sunflower seed oil were fed. Thus, it would appear that soybean oil has a higher metabolizable energy content per se than other fat sources tested or that soybean oil exerts an influence on other ration ingredients whereby ration metabolizable energy is increased over that realized when other fat sources are used. But, although soybean oil significantly increased ration metabolizable energy as compared with other fat sources tested, soybean oil did not consistently stimulate growth any more than did comparable levels of animal tallow, rapeseed oil and sunflower seed oil.

The fatty acid composition of the dietary fat sources and the abdominal adipose tissue are presented in table 3. In general, fatty acid composition of the adipose tissue reflected that of the dietary fat, particularly when an 8% level of the fat source was used. Cruickshank ('34), Adamson et al. ('61) and Rogler and Carrick ('61) reported that, on the basis of iodine number, degree of unsaturation of dietary fatty acids had a marked influence on carcass fat of chickens. Feigenbaum and Fisher ('59) using gas chromotography, also observed that the fatty acid composition of carcass fat was influenced by the composition of the dietary fat.

The data presented herein show that in comparison with the adipose tissue of chicks receiving the basal ration, adipose tissue of chicks receiving animal tallow reflected the relatively high stearic-oleic acid ($C_{18} + C_{18:1}$) and low linoleic acid ($C_{18:2}$)

TABLE 2

Metabolizable energy of rations containing different fat sources as determined with cockerels from 4 to 8 weeks of age

	Ration treatment[1]								
	Basal (no added fat)	4% Rapeseed oil	4% Animal tallow	4% Sunflower seed oil	4% Soybean oil	8% Rapeseed oil	8% Animal tallow	8% Sunflower seed oil	8% Soybean oil
Metabolizable energy, Cal./gm dry ration	2.89 ± 0.02[2]	2.99 ± 0.02	3.04 ± 0.03	3.11 ± 0.01	3.22 ± 0.02	3.25 ± 0.07	3.26 ± 0.01	3.29 ± 0.02	3.45 ± 0.04

[1] All means not underscored by the same line are significantly different at $P < 0.05$.
[2] Figures preceded by ± sign are standard errors of the mean.

TABLE 3

Fatty acid composition of fat sources used in rations and abdominal adipose tissue of 8-week-old chicks

	C_{14}	$C_{16}+C_{16:1}$	$C_{18}+C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:1}$	$C_{22:1}$
	Fatty acid composition of fat sources (percentage of total methyl esters)						
Fat source added to ration							
Animal tallow	3.75	33.14	61.88	1.23	—	—	—
Soybean oil	—	11.11	28.87	52.44	7.58	—	—
Rapeseed oil	4.30	28.57	20.09	6.50	5.34	3.72	31.48
Sunflower seed oil	—	6.80	20.93	70.47	1.80	—	—
	Fatty acid composition of abdominal adipose tissue (percentage of total methyl esters)						
Ration treatments							
Basal (no added fat)	1.51 ± 0.33[1]	34.79 ± 1.26	47.62 ± 0.61	16.08 ± 1.05	—	—	—
Animal tallow, 4%	2.32 ± 0.34	33.25 ± 2.10	53.46 ± 2.11	10.97 ± 1.34	—	—	—
Animal tallow, 8%	2.18 ± 0.36	34.64 ± 0.39	55.43 ± 1.18	7.75 ± 0.61	—	—	—
Soybean oil, 4%	2.85 ± 0.51	29.72 ± 3.24	52.58 ± 1.46	14.85 ± 2.61	0.75 ± 0.29	—	—
Soybean oil, 8%	1.07 ± 0.80	21.83 ± 2.24	44.04 ± 4.40	33.06 ± 6.15	1.60 ± 0.21	—	—
Rapeseed oil, 4%	1.45 ± 0.48	26.51 ± 1.35	49.60 ± 4.29	11.65 ± 3.55	0.98 ± 0.32	5.10 ± 0.39	4.71 ± 0.33
Rapeseed oil, 8%	0.96 ± 0.57	17.36 ± 1.00	48.47 ± 2.94	17.73 ± 5.06	1.34 ± 0.26	8.06 ± 0.88	6.08 ± 0.98
Sunflower seed oil, 4%	3.27 ± 0.20	28.44 ± 0.97	51.42 ± 2.22	16.13 ± 3.29	—	—	—
Sunflower seed oil, 8%	1.30 ± 0.32	19.05 ± 1.00	36.69 ± 5.90	41.36 ± 7.28	—	—	—

[1] Figures preceded by ± sign are standard errors of the mean.

fractions supplied by the fat source. Inclusion of 8% of soybean oil or sunflower seed oil in the ration caused a marked decrease in the palmitic-palmitoleic acid ($C_{16} + C_{16:1}$) fraction of the adipose tissue with a simultaneous increase in the linoleic acid fraction. The presence of substantial portions of eicosenoic ($C_{20:1}$) and erucic ($C_{22:1}$) acids in adipose tissue of chicks receiving dietary rapeseed oil is further evidence for an apparent direct deposition of some unaltered dietary fatty acids in body fat stores. The influence of rapeseed oil on body fat of fowl had been reported by Chomyszyn ('55). He observed that, after administering 40 ml of rapeseed oil per day for 4 successive days, the subcutaneous and internal fat of young geese assumed the characteristics of rapeseed oil (iodine number, M.P. and odor).

The influence of dietary fatty acids on the carcass fat is not a unique occurrence. Horlich and O'Neil ('58) and Feigenbaum and Fisher ('59) observed that fatty acids of egg yolk could also be altered by degree of unsaturation of dietary fatty acids and Beare et al. ('61) showed that the milk fat from rats fed rapeseed oil contained eicosenoic and erucic acids, although in lesser quantities than present in the original oil.

The results of the present study indicate that the composition of the dietary fat may be an important consideration in the production of chicken carcasses for human consumption. Change in the physical characteristics of the carcass fat, and influence on cooking loss, development of rancidity and off-flavors due to various dietary fats are factors that require further study.

SUMMARY

Chickens were fed from zero to 8 weeks of age, rations containing 4 or 8% of animal tallow, soybean oil, rapeseed oil or sunflower seed oil. As compared with a ration with no added fat, only the inclusion of 4% of animal tallow in the ration failed to significantly increase weight gain. In general, all fat additions markedly improved efficiency of feed utilization. The use of equivalent levels of animal tallow, rapeseed oil and sunflower seed oil resulted in ration metabolizable energy values which were approximately equal. However, the metabolizable energy content of rations containing soybean oil was significantly higher than that of rations containing comparable levels of other fat sources. Fatty acid analysis by gas chromatography showed that the composition of chick adipose tissue reflected that of the dietary fat. The presence of substantial portions of eicosenoic and erucic acids in adipose tissue of chicks receiving dietary rapeseed oil provides additional evidence for an apparent direct deposition of some unaltered dietary fatty acids in the body fat stores.

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Nutritional and Microbial Effects on Liver Monoamine Oxidase and Serotonin in the Chick[1]

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Much interest exists in the physiological role of monoamine oxidase, its inhibitors and their therapeutic applications (Zeller, '51; Blaschko, '57; Davison, '58). The present study was undertaken with the thesis that bacterial amines absorbed from the intestinal tract should induce the synthesis of monoamine oxidase in host tissues, and thereby influence serotonin and adrenalin levels. In contrast, the intestinal flora might reduce the liver monoamine oxidase of a host, as a result of substrate inhibition of enzyme. This report describes the effects of microbial contamination and nutritional status on liver monoamine oxidase and serotonin in chicks.

EXPERIMENTAL

Germfree White Leghorn chicks were obtained and reared by procedures previously described (Phillips et al., '60)[2] except that the present isolators were equipped with glass wool filters[3] and sterilized with peracetic acid solution (Trexler and Reynolds, '57). Fertile eggs were obtained from local hatcheries, and sterilized by immersion in 2% $HgCl_2$ solution at 38°C for 10 min. Approximately equal numbers of both sexes of chicks were used in all experiments. Except as noted, control chicks were treated and reared under the same conditions as germfree chicks; control isolators were not sterilized. Chicks were reared under fluorescent lights for 12 hours during each 24-hour day; the darkness cycle was 6 PM to 6 AM. The term "germfree" chicks in this context does not exclude contamination by viruses or rickettsiae. The bacteriological routine was essentially that described by Wagner ('59); and at autopsy the intestinal contents of germfree and monocontaminated chicks were analyzed for bacterial contamination.

The following diets were used. Diet A was similar to a commercial growing mash.[4] Diet B was the purified casein-starch diet of Forbes and Park ('59), with which they obtained excellent growth of germfree chicks. Diet C was a pyridoxine test[5] diet (Ott, '46). Diet A had the following percentage composition: ground corn meal, 30; ground oats, 10; alfalfa meal, 5; wheat middlings, 10; soybean meal, 23; meat and bone scraps, 5; casein, 5; corn oil, 1; salt mixture (Reyniers et al., '50), 8; cod liver oil, 1.13; and vitamin mixture, 1.13.[6] The two latter supplements contributed the following quantities of vitamins per 100 gm of diet: vitamin A, 960 USP units; vitamin D, 96 USP units; and (in milligrams) α-tocopherol, 6; menadione, 3; *p*-aminobenzoic acid, 6; niacin, 5; riboflavin, 1; pyridoxine·HCl, 1; thiamine·HCl, 1; Ca pantothenate, 3; choline, 75; inositol, 6; also (in μg) biotin, 20; folic acid, 100; and vitamin B_{12}, 2. Diet A had 17% of protein, 3% of fat, 7% of fiber and 48% N.F.E.

Monoamine oxidase was determined on fresh tissues of animals killed by decapitation. Weighed tissues were homogenized in cold (0° C) Sorensen buffer at pH 7.4 and the enzyme assayed in diluted homogenates by a modification of the method of Cotzias and Dole ('51) and Cotzias et al. ('54); tyramine hydrochloride was the substrate. The reaction mixture was aerated with pure oxygen during incubation, as

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[2] Rupp, F. A. 1960 Influence of *Clostridium perfringens* on liver monoamine oxidase in chicks. Ph. D. Thesis, Syracuse University.

[3] American Sterilizer Co., Erie, Pennsylvania.

[4] Grange League Federation (GLF), Ithaca, New York.

[5] General Biochemicals, Inc., Chagrin Falls, Ohio.

[6] Nutritional Biochemicals Corporation, Cleveland, Ohio.

suggested by Kapeller-Adler ('51); ammonia was recovered (Sobel et al., '47) and determined colorimetrically using a phenol hypochlorite reagent. Total liver monoamine oxidase activity was expressed as milligrams of N liberated from the substrate per kilogram of body weight, after appropriate corrections for ammonia production in tissue without added substrate. Serotonin was assayed on fresh tissues after Weissbach et al. ('58).

In some experiments, *Clostridium perfringens*[7] type A, strain EIG-7, was fed to conventional chicks. The organism was first grown in thioglycollate broth (Difco) for 18 hours at 37°C; 20 ml of this culture was added to the diet and water of 10 chicks. This procedure was repeated every 48 hours during the first week after hatching, and once during the second week.

RESULTS AND DISCUSSION

The total liver monoamine oxidase in conventional chicks displayed an abrupt increase of about threefold between the 6th and 14th days of age (table 1). Because similar data are not available for the germfree chick at one week, it is not known whether this sharp rise in liver enzyme activity is the result of developmental changes, nutritional or microbial effects. From the second through the 4th week, liver monoamine oxidase was relatively constant in conventional chicks; total enzyme was somewhat less in older birds.

At two weeks of age, the liver enzyme level was significantly higher in germfree than in conventional birds; both groups of chicks were comparable in their general appearance and growth (table 2). Unsuccessful attempts were made to demonstrate a growth promotion effect by feeding oxytetracycline to chicks for two weeks, in the presence and absence of *Cl. perfringens* in the diet or water. Liver monamine oxidase levels were not influenced by feeding the antibiotic (table 2).

Germfree chicks at 4 weeks of age and having a tremors syndrome, had almost twice the liver enzyme activity of conventional birds (table 3). Chicks that were germfree for three weeks and then accidently monocontaminated with a *Bacillus* species, had an enzyme level that was intermediate between that of the germfree and control birds. The growth rate and appearance of the germfree chicks shown in table 3 were considerably inferior to those of the control group, especially after the second week. Indeed, these germfree chicks, as well as chicks monocontaminated in the 4th week with the above *Bacillus* sp., manifested some of the characteristics of an encephalo-meningeal syndrome of germfree chicks described by Gordon et al. ('59). We have encountered this syndrome only during May to August; it has occurred in birds fed the purified diet B as well as crude diet A. The syndrome was not apparent in chicks reared in December to April. Multi-contaminated chicks did not display the syndrome at any time. The ailment could not be traced to irradiation of the diet, mercuric chloride poisoning, or faulty air supply to the isolators. On the assumption that the above syndrome might have been due to a virus infection, chicks were infected with Newcastle disease. This infection did not influence liver monoamine oxidase.

[7] Kindly provided by Drs. M. Forbes and M. Lev, Walter Reed Army Institute of Research.

TABLE 1

Age dependence of liver monoamine oxidase in White Leghorn chicks[1]

Age	No. of chicks	Body weight	Liver weight	Total monoamine oxidase
days		*gm*	*as % of body weight*	*mg N/kg body weight*
6	4	52 ± 4	3.3 ± 0.2	4.5 ± 1.4
9	11	65 ± 6	3.2 ± 0.3	6.6 ± 1.9
14	18	99 ± 11	3.0 ± 0.3	16.0 ± 3.8
28	27	275 ± 39	2.7 ± 0.6	16.0 ± 2.9
56	5	578 ± 40	2.4 ± 0.1	11.8 ± 1.1
84	5	1006 ± 57	1.9 ± 0.1	12.6 ± 1.8

[1] Conventional birds were fed diet A (in text), a crude growing mash with no added antibiotics. Values are means ± SD.

TABLE 2

Liver monoamine oxidase in germfree and conventional White Leghorn chicks at two weeks

Group	Treatment[1]	No. of chicks	Body weight	Liver weight	Total monoamine oxidase
			gm	*as % of body weight*	*mg N/kg body weight*
Reared in isolators					
1	*Cl. perfringens*	10	99 ± 19[2]	3.4 ± 0.7	25.7 ± 4.6
2	Control	11	97 ± 11	2.9 ± 0.2	15.8 ± 3.5
Reared in conventional brooders					
3	*Cl. perfringens*	10	98 ± 10	3.2 ± 0.3	17.3 ± 2.9
4	*Cl. perfringens* + OTC	10	113 ± 13	3.0 ± 0.2	15.2 ± 2.6
5	OTC	9	103 ± 8	3.1 ± 0.1	15.2 ± 4.2
6	Control	7	91 ± 8	3.2 ± 0.4	16.3 ± 4.2

Significance of differences in monoamine oxidase

Groups compared	*t*	*P*
1–2	5.3	< 0.01
3–4	1.6	< 0.20
3–5	1.2	< 0.30

[1] Diet A in text; oxytetracycline (OTC) added in diet at 60 ppm where specified. *Clostridium perfringens* added in diet or drinking water (see text).
[2] Mean ± SD.

TABLE 3

Liver monoamine oxidase at 4 weeks in germfree White Leghorn chicks with a tremors syndrome

Group	Treatment[1]	No. of chicks	Body weight	Liver weight	Total monoamine oxidase
			gm	*as % of body weight*	*mg N/kg body weight*
A	Germfree	22	131 ± 25[2]	2.5 ± 0.2	29.4 ± 3.2
B	*Bacillus sp.*[3]	8	199 ± 29	2.7 ± 0.3	24.1 ± 2.6
C	Control	27	275 ± 39	2.7 ± 0.6	16.0 ± 2.9

Significance of differences by *t* test

Groups compared	t	P
A–C	14.7	< 0.01
A–B	4.1	< 0.01
B–C	6.9	< 0.01

[1] Diet A in text. All chicks were reared in isolators. Experiments conducted during April to July, 1960. Illness occurred in groups A and B after the 2nd week: no illness was noted in group C.
[2] Mean ± SD.
[3] Germfree chicks were accidentally monocontaminated in the 4th week.

Germfree chicks without the above syndrome, at 4 weeks contained more total liver monoamine oxidase than conventional control animals (table 4). Conventional chicks fed purified diet B for 4 weeks, weighed considerably less than chicks receiving crude diet A (tables 3, 4). A comparison of data in tables 3 and 4 suggests that the tremors syndrome may have enhanced liver enzyme activity of germfree chicks.

Both mild and severe pyridoxine deficiencies in conventional chicks resulted in elevated liver monoamine oxidase (table 5). A similar, more variable response was observed in the liver of B_6-deficient rats; and the requirement for pyridoxine in the terminal metabolism of monoamine oxidase was given as a possible explanation (Sourkes, '58). Another possibility although not established, is that pyridoxine, or an associated metabolite, inhibits the

TABLE 4

Liver monoamine oxidase in healthy germfree White Leghorn chicks at 4 weeks

Group	Treatment[1]	No. of chicks	Body weight	Liver weight	Total monoamine oxidase
			gm	*as % of body weight*	*mg N/kg body weight*
A	Germfree	14	159 ± 38[2]	3.3 ± 0.7	19.7 ± 2.4
B	Control	13	171 ± 47	3.4 ± 0.4	13.6 ± 1.1

Significance of differences by *t* test

Groups compared	*t*	*P*
A–B	7.9	< 0.01

[1] Diet B in text. All chicks were reared in isolators. Experiments conducted during January to April, 1961.
[2] Mean ± SD.

TABLE 5

Effect of starvation and pyridoxine deficiency on liver monoamine oxidase in White Leghorn chicks at 30 days

Group	Treatment	No. of chicks	Body weight	Liver weight	Total monoamine oxidase
			gm	*as % of body weight*	*mg N/kg body weight*
Experiment 1[1]					
A	Starved	9	202 ± 32[2]	2.4 ± 0.2	12.2 ± 1.8
B	Vitamin B_6-deficient	8	202 ± 21	2.7 ± 0.3	17.6 ± 1.0
C	Controls	8	279 ± 39	2.9 ± 0.3	12.8 ± 2.5
Experiment 2[3]					
D	Vitamin B_6-deficient	6	98 ± 8	4.0 ± 0.6	22.2 ± 2.4
E	Controls	10	137 ± 24	3.6 ± 0.4	13.2 ± 1.5

Significance of differences by *t* test

Groups compared	*t*	*P*
A–C	0.52	< 0.70
A–B	7.15	< 0.01
B–C	4.75	< 0.01
D–E	8.55	< 0.01

[1] Chicks were fed diet A during first three weeks. Diet C was supplemented with 5 μg of pyridoxine/gm of diet and fed during the fourth week, except that birds in group B received no pyridoxine. Chicks of group A were starved for 72 hours prior to sacrifice.
[2] Mean ± SD.
[3] Chicks were given diet C (in text). Groups D and E were fed pyridoxine at 0.5 and 2.5 μg/gm of diet, respectively. In group D, 5 of the initial group of 11 chicks died before 30 days.

enzyme. Pyridoxamine inhibits diamine and spermine oxidases (Sourkes, '58). Conventional chicks showed a sharp loss in weight due to starvation for 72 hours, but their liver enzyme level was not significantly different from control animals (table 5).

Conventional chicks fed a pyridoxine-deficient diet for 30 days had significantly lower liver serotonin values than control birds. The livers of vitamin B_6-deficient chicks and their controls contained 0.5 ± 0.00 and 1.4 ± 0.05 μg (± SD) of serotonin per gm of fresh tissue, respectively (data on chicks in table 5, exp. 2). Statistical treatment of the above data resulted in $t = 20.7$ and $P < 0.01$. Pyridoxine deficiency was earlier reported to reduce serotonin concentration in the liver of chicks, because the vitamin is a co-factor for 5 - hydroxytryptophane decarboxylase (Weissbach et al., '57). In our studies, liver serotonin concentrations were similar in germfree and conventional chicks. The

liver serotonin values of conventional chicks that were starved for 72 hours were comparable to those of birds not starved.

The ratios of liver to body weights appeared slightly higher in chicks fed purified diets (B and C) for 4 weeks than in chicks fed the crude diet A (tables 3, 4, 5). The above ratios were similar in germfree and conventional chicks (tables 3, 4).

No information is available from these studies regarding factors responsible for the apparent inhibition of liver monoamine oxidase in chicks contaminated with microorganisms. Work is in progress on the identification of microbial products, including amines, absorbed in the liver from the intestinal tract; and the compounds will be examined for inhibition of the liver enzyme.

SUMMARY

The total liver monoamine oxidase in germfree chicks was significantly higher than in conventional (contaminated) animals. Chicks monocontaminated with a *Bacillus* sp., manifested a liver enzyme value that was intermediate between the two above groups of animals. The concentrations of liver serotonin were similar in germfree and conventional chicks. A pyridoxine deficiency in conventional birds reduced liver serotonin, and elevated liver monoamine oxidase activity. Possible explanations of these results were discussed.

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Vitamin K Deficiency in the Baby Pig

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Contrary to previous belief, vitamin K has been shown to be an essential nutrient for the rat without the presence of any bacteriostatic agent in the diet and with coprophagy permitted (Mameesh and Johnson, '59). The requirement for vitamin K_1 (3-phytyl-2-methyl-1,4-naphthoquinone) has also been reported by these investigators to be 0.1 μg per gm of diet for non-coprophagic rats fed a synthetic vitamin K-free diet.

Vitamin K deficiency has been recognized as a clinical entity in the newborn infant for several years. We have considered the baby pig to be a useful experimental animal in pilot studies for the human and have used the baby pig in nutritional studies of vitamin B_{12}, vitamin E, vitamin A, choline and folic acid. In this paper we present the first report of vitamin K deficiency in this species.

EXPERIMENTAL

One- and two-day-old baby pigs obtained from a commercial source were used in these experiments. The animals were housed individually in wire-bottom metal cages and kept in a laboratory in which the temperature was maintained at 85°F for the first three weeks, after which it was slowly decreased to 75°F. The animals were grouped according to size and litter, and the treatments randomly assigned. Eleven baby pigs were used in the first experiment: 6 animals received the complete basal diet (table 1) and 5 received the vitamin K-free diet. Coprophagy was reduced by using raised wire-bottom cages which were cleaned daily. Phthalylsulfathiazole and oxytetracycline were fed throughout the experiments as prophylactics.

Feed intake was restricted to 400 ml or less per day during the first 10 days to reduce the possibility of diarrhea which might result from overfeeding. Thereafter the animals were fed ad libitum 4 times daily. The feeding dishes were washed thoroughly with hot water and a household detergent before each feeding. Cages were thoroughly scrubbed each day. The cages were also steamed once a day during the first three weeks. Feed records were kept and the animals were weighed every third day, 3.5 hours after the 8:00 AM feed.

In the second experiment additional efforts were made to eliminate possible sources of vitamin K for the animal. Entirely synthetic glycerol trioleate[2] and methyl linoleate[3] which did not contain any vitamin K or require lecithin for emulsification were used throughout the experiment. The amount of coprophagy was reduced by cleaning the cages 4 times a day or after each feed. The levels of phthalylsulfathiazole and oxytetracycline were also increased 1.5- and 3-fold (to 5 gm per liter and 60 mg per kg of dry matter of diet, respectively). During the latter part of the experiment, 4 animals were housed in small coprophagy-prevention cages which confined the animals and prevented them from having access to their feces. The feeding and care of the animals was in other respects similar to that described by Johnson et al. ('48).

"Activated clotting times" were measured, using a desiccated thromboplastin preparation made from rabbit brain tissue,[4] by the one-stage procedure (Smith et al., '39; Hoffman et al., '42; Schwager and Jaques, '49; Thompson, '60) as modified for micro quantities of whole blood by Glover and Kuzell ('61). The latter investigators have standardized the conditions

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[2] K and K Laboratories, Inc., Jamaica, New York.
[3] Nutritional Biochemicals Corporation, Cleveland.
[4] Difco Laboratories, Detroit 1, Michigan.

TABLE 1

Composition of basal diet[1]

Constituent[2]	Amount
	% dry matter
Protein: soya protein[3]	34.05
methionine	0.49
Carbohydrate: glucose[4]	39.83
Fat	11.70
Ash	13.93

Minerals	*mg/l*	Vitamins[5]	*mg/l*
Sodium phosphate (monobasic)	7.75	Thiamine·HCl	1.01
Calcium hydroxide	3.80	Riboflavin	2.02
Citric acid	3.49	Pyridoxine·HCl	2.02
Calcium chloride	2.95	Nicotinic acid	4.03
Potassium hydroxide	1.51	Ascorbic acid	24.80
Sodium hydroxide	1.47	Choline chloride	201.50
Potassium phosphate (monobasic)	1.43	Calcium pantothenate	12.09
Magnesium oxide	0.48	*p*-Aminobenzoic acid	4.03
Ferric citrate	0.36	Biotin	0.016
Zinc chloride	0.022	Pteroylglutamic acid	0.081
Manganese sulfate	0.019	Cyanocobalamin[6]	0.8 μg/kg/day
Potassium iodide	0.011	Vitamin A	3,000 IU
Calcium fluoride	0.006	Vitamin D_2	300 IU
Copper sulfate	0.004	α-Tocopheryl succinate	1.5 mg
Fat			
Glycerol trioleate[7]	16.67	Phthalylsulfathiazole	2 gm
Methyl linoleate	2.44	Oxytetracycline	20 mg/kg DM[8]
Lecithin[9]	0.44		

[1] Thiamine·HCl, riboflavin, pyridoxine·HCl, Ca pantothenate, nicotinic acid, cyanocobalamin, pteroylglutamic acid, menadione, biotin, α-tocopheryl succinate, *p*-aminobenzoic acid and sulfathalidine (phthalylsulfathiazole) were generously supplied by Merck, Sharp and Dohme, Rahway, New Jersey, through the courtesy of Drs. David F. Green and S. F. Scheidy. Methionine was generously supplied by Dow Chemical Company, Midland, Michigan, through the courtesy of Dr. Julius Johnson. Water-soluble vitamin A and vitamin D were generously supplied by Endo Products Company, Richmond Hill, New York, through the courtesy of Dr. S. M. Gordon. Terramycin hydrochloride (oxytetracycline) was supplied by Pfizer and Company, New York, through the courtesy of Dr. J. H. Hare. Lecithin was supplied by Central Soya Company, Chicago, through the courtesy of Dr. Edwin Meyer.

[2] The synthetic milk containing 16.7% solids and 2% fat was prepared with slight modification according to the procedure of Clark ('27).

[3] ADM C-1 Assay Protein, Archer-Daniels-Midland Company, Cincinnati.

[4] Cerelose, Corn Products Company, New York.

[5] The "water-soluble" vitamins were administered in a 30% ethanolic solution at the time of feeding. Water-soluble preparations of vitamins A and D as well as crystalline vitamin E were added to each 10 gallons of milk as it was fed.

[6] Cyanocobalamin and vitamin K for the positive controls (as 50 μg menadiol sodium diphosphate/kg body weight) were administered intramuscularly once a week.

[7] The ratio of trioleate to linoleate in the diet was 3 to 0.44.

[8] Oxytetracycline concentration was increased to 60 mg/kg of dry matter of diet in the second experiment.

[9] Lecithin (as 0.2% of the fat) was added to stabilize the fat emulsion in the first experiment.

for the procedure and report a correlation coefficient of 0.96 for "activated clotting time" as measured by their method and the "prothrombin time" of Quick ('39). The two terms, activated clotting time and prothrombin time are used synonymously in this manuscript. Measurements were made on freely flowing blood taken immediately after lancing an ear vein. Simultaneous measurements of blood obtained by cardiac puncture and from the ear vein in one animal indicated prothrombin times that were identical.

Hematocrits were calculated from measurements of packed cell volume in oxalated capillary tubes. Blood from the lanced ear vein was drawn up into the capillary tube, sealed with plicene cement and centrifuged.

The response to vitamin K was studied after both the oral and intramuscular administration of an aqueous solution of menadiol sodium diphosphate (the tetrasodium salt of the diphosphoric acid ester of 2-methyl-1,4-naphthohydroquinone).

Postmortem examinations were carried out on all animals that died.[5]

[5] All postmortem examinations were performed under the direction of Dr. E. I. Pilchard, Director of the Illinois State Department of Agriculture Research and Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois, Urbana.

RESULTS

1. Chronic experiment. The results of the first experiment are tabulated in tables 2 and 3. Four of the 5 animals that received the vitamin K-deficient diet developed symptoms. These symptoms included a hypersensitive skin, a significantly increased prothrombin time and finally anorexia and weakness. In this experiment the mean prothrombin time of 22.6 seconds (range 21 to 27 seconds) for the vitamin K-deficient animals was significantly higher ($P < 0.01$) than the mean time of 14 seconds (range 12 to 16 seconds) for the 6 control animals. The mean prothrombin time for the three treated animals fell to 15 seconds (range 14 to 16 seconds). Although the increase in prothrombin time was not nearly as great in this experiment as has been observed in the vitamin K-deficient rat, it occurred concomitantly with the other symptoms.

Two deficient animals were treated during the course of the experiment and responded both to orally and intramuscularly administered vitamin K. The response was very dramatic in the animal that received 1 mg of menadiol sodium diphosphate intramuscularly. Whereas this animal had been unable to stand and eat, it was up and rather active within three hours after the injection. Protection was maintained for 6 days, after which the health of the animal deteriorated again and it died on the 7th day after treatment. The oral administration of 0.6 mg of menadione over a period of 36 hours (200 μg per kg of body weight per day over 5 feeding periods) brought the second pig to its feet and afforded protection for 5 days.

The three remaining animals that received the vitamin K-deficient diet were left untreated. One of these developed symptoms and died on the 27th day. Another pig developed symptoms on the 27th day and spontaneously recovered after it began to consume its feces. The third animal failed to demonstrate any symptoms.

TABLE 2

Clinical summary of baby pigs fed the vitamin K-deficient diet in experiment 1

Pig no.	Day symptoms[1] appeared and animal treated	Treatment with menadiol sodium diphosphate	Response	Final outcome
			hours	
18	24th	Orally 200 μg/kg/day over 6 feeds	24	Spontaneous recovery (eating stools, 32nd day)
1	24th	Intramuscularly 150 μg/kg	3	Died, 31st day
21	26th	Untreated	—	Died, 27th day
27	27th	Untreated	—	Spontaneous recovery (eating stools, 30th day)
14	None	—	—	—

[1] Symptoms included a prolonged prothrombin time, hypersensitivity, anemia and finally anorexia and weakness.

TABLE 3

Prothrombin times of baby pigs fed the vitamin K-deficient diet in experiment 1

Pig	No. of animals	Whole blood prothrombin time in seconds (mean ± SD)	Probability of difference
Positive controls	6	14.0 ± 1.32	$P < 0.01$
Vitamin K-deficient	5	22.6 ± 2.93	
Vitamin K-deficient, treated with menadiol sodium diphosphate	3	15.0	

Menadiol sodium diphosphate was administered intramuscularly to the three surviving animals that had received the vitamin K-free diet at approximately 5 weeks of age. This treatment was without benefit and confirmed the spontaneous remission of the deficiency.

Body weight or food intake does not reflect vitamin K deficiency. The effects of the deficiency are very sudden or toxic and cause death before a change in growth can be observed. When the deficiency symptoms became apparent (end of 4th week) the body weight for the 5 deficient and 6 control piglets was observed to be 3.06 ± 0.70 and 3.36 ± 0.42 kg, respectively. Postmortem examination of the two vitamin K-deficient animals that died revealed no evidence of internal hemorrhages.

2. *Acute experiment.* The results of this experiment are tabulated in table 4 and summarized in figure 1. The purpose of this second experiment was (1) to make several refinements in the diet, rendering it more nearly free of vitamin K; (2) to accumulate data on the minimal curative injected dose and the oral requirement; and (3) to make other observations on various aspects of the deficiency syndrome: effect of high levels of phthalylsulfathiazole in the diet, housing in coprophagy-preventing cages and the incidence of anemia in this syndrome.

It had become obvious in the first experiment that clinical response was a poor indication of the vitamin K status of the animal. Therefore, in the second experiment we used prothrombin time as the principal sign of deficiency rather than as a confirmatory test. Prothrombin times were measured, on the average, every two to three days for the deficient group and every 5th day for the controls.

In this experiment, where prolonged prothrombin time was used as the criterion to establish vitamin K deficiency, every animal fed the vitamin K-free diet became deficient. The prothrombin level in the blood of all 10 of the vitamin K-deficient piglets decreased by the third week. This decrease in prothrombin level was reflected in a highly significant increase ($P < 0.001$) in the mean prothrombin time from 14.4 to 19.7 seconds by the 22nd day. The mean prothrombin time for the vitamin K-deficient group was 49.6 seconds by the 35th day. Prothrombin times greater than 120 seconds were recorded for 4 animals: two by the 16th day and two by the 35th day. One of these animals died of hemorrhage from the small cut of the ear vein

TABLE 4

Prothrombin response of vitamin K-deficient piglets to intramuscular and oral administration of menadiol sodium diphosphate

	Mean prothrombin time			
	Positive control	Vitamin K-deficient		
		Before treatment	After treatment	Menadione dosage
No. of animals	5	10		
At start of experiment	13.9 sec.	14.4 sec.		
At 3 weeks		19.7 sec.[1]		
At 5 weeks	16.6 sec.	58.7 sec.	17.4 sec.[2]	IM 50–400 μg/kg (one injection)
At 6 weeks	14.5 sec.	21.0 sec.	15.7 sec.[1]	IM 20–40 μg/kg (one injection)
At 8 weeks	14.3 sec.	18.0 sec.	15.7 sec.[3]	Orally 5 μg/kg/day (6 days)

[1] $P < 0.001$.
[2] $P < 0.02$.
[3] $P = 0.05$.

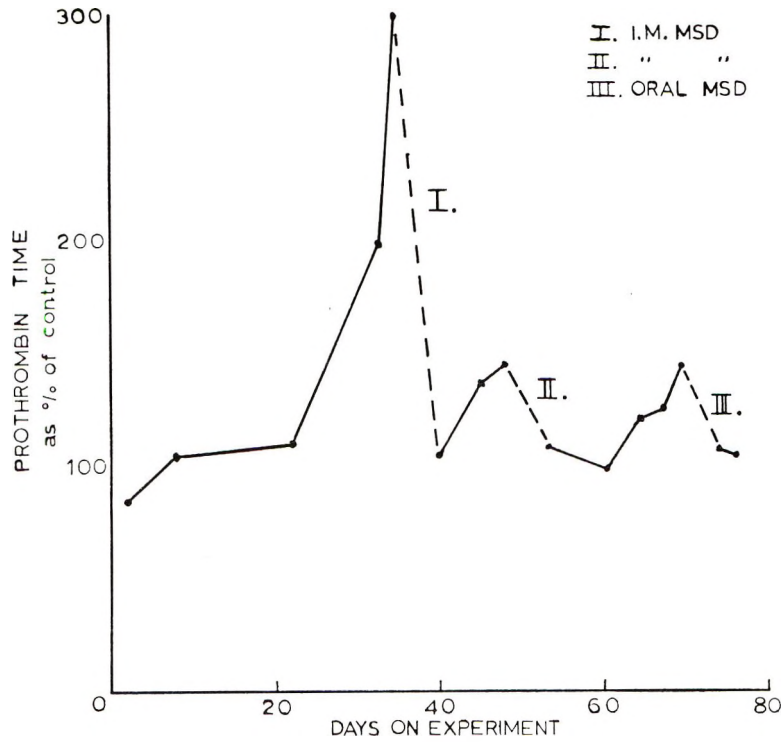

Fig. 1 Prothrombin response of vitamin K-deficient piglets to intramuscular and oral administration of menadiol sodium diphosphate (MSD).

on the 33rd day. Subsequently these small cuts were cauterized with a hot rod for any animals that did not stop bleeding within an hour. None of the animals developed clinical signs of deficiency in these short-term studies.

Tests were conducted on the 35th and 42nd days to define the minimal curative injected dose for vitamin K. During the first test, dosages of 50 to 400 μg of menadiol sodium diphosphate per kg of body weight reduced the activated clotting time from 58.7 to 17.4 seconds ($P < 0.02$) within 4 hours. During the second test, dosages of 20, 30 and 40 μg per kg of body weight again reduced clotting times from 21.0 to 15.7 seconds ($P < 0.001$) within 4 hours. These data indicate that the minimal curative injected dose for this compound is less than 20 μg per kg of body weight.

Data concerning the dietary requirement for vitamin K was also accumulated, using the change in prothrombin time as the criterion. Prothrombin times had risen again by the 8th week after the second curative dose test. At that time the oral administration of 5 μg of menadiol sodium diphosphate per kg of body weight per day to 4 of the deficient animals was begun and was continued for one week. The prothrombin time of each of these animals decreased, with a mean decrease from 18.0 to 15.7 seconds ($P = 0.05$).

The effect of a dietary bacteriostatic agent on vitamin K deficiency was observed in two short-term experiments. The mean prothrombin time of 7 deficient animals, which did not receive phthalylsulfathiazole for 7 days, and again later for 12 days, changed from 18.1 to 21.7 seconds. The mean prothrombin time of 6 animals that did receive phthalylsulfathiazole over these periods increased from 16.9 to 20.7 seconds.

Additional short-term studies on the effect of intestinal synthesis on vitamin K deficiency were conducted using coprophagy-preventing cages. Four deficient animals were housed in such cages for periods

of 10 to 30 days. Their mean prothrombin time changed from 17.6 to 19.6 seconds as compared with the change of 17.0 to 17.8 seconds over the same period for 5 vitamin K-deficient animals housed in the wire-bottom cages.

Since the blood of the vitamin K-deficient animals always appeared thin and watery, data concerning anemia were accumulated during the 8th week. The mean hematocrit value for the 6 control pigs was 42.7% as compared with 29.6% ($P=0.05$) for the 7 deficient animals. During treatment with dietary vitamin K, the hematocrit for the deficient group increased to 37.5% ($P < 0.05$).

DISCUSSION

In these experiments data were accumulated demonstrating the need for vitamin K by baby pigs fed a "synthetic" liquid diet. Fourteen out of 15 animals developed symptoms which included a highly significant increase in prothrombin or activated clotting time, hypersensitivity, anemia and finally anorexia and weakness. The animals developed symptoms and died by the 4th or 5th week when fed this vitamin K-deficient diet. Although these symptoms are reminiscent of sulfonamide toxicity, they were observed in the first experiment where the concentration of phthalylsulfathiazole in the diet could not be considered toxic. Furthermore, the oral or intramuscular administration of vitamin K (menadiol sodium diphosphate) produced alleviation of these symptoms within two to 4 hours.

The failure of symptoms to develop in one of the piglets and to disappear in two others in the first experiment may have resulted from several causes. Analysis by the chick bioassay[6] revealed that the emulsifier used (lecithin)[7] provided approximately 4 μg of vitamin K_1 activity per 100 gm of diet, or roughly half the estimated amount of vitamin K_1 the pig would require based on the rat's requirement of 10 μg per 100 gm of diet (Mameesh and Johnson, '60). A second source of vitamin K could have been the more crude glycerol trioleate[8] which was present in the diet from days 29 to 39. Since the pens were cleaned only once each day in the first experiment, it is probable that coprophagy also provided some vitamin K to the animals.

In the second experiment, where these factors were all considered, each of the 10 animals in the vitamin K-deficient group developed very prolonged prothrombin times, the difference between the mean of the deficient group and that of the controls was highly significant.

The indication in these data that housing in coprophagy-preventing cages or the exclusion of high levels of sulfathalidine from the diet has little effect on prothrombin time probably means that the thorough cleaning of the feeding pans and cages 4 times each day was effective in reducing coprophagy. Such procedures do not appear to be necessary for producing vitamin K deficiency in newborn pigs. Pigs will develop deficiency symptoms and die before they begin to practice coprophagy.

SUMMARY

Vitamin K has been shown to be an essential nutrient for newborn pigs housed in raised wire-bottom cages and fed a "synthetic" liquid diet. Fourteen out of 15 animals developed symptoms which include a highly significant increase in prothrombin time, hypersensitivity, anemia and, finally, anorexia and weakness. Whereas the animals died by the 4th or 5th week if they did not receive some source of this vitamin, they responded clinically and biochemically to the oral or intramuscular injection of vitamin K (menadiol sodium diphosphate) in two to 4 hours. Using prothrombin time as the criterion, the dietary requirement of newborn pigs for this compound is approximately 5 μg per kg of body weight per day, and the minimal curative injected dose is something less than 20 μg per kg of body weight.

Other observations on the occurrence of internal hemorrhage and the effect of coprophagy and a dietary bacteriostatic agent were also made.

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[6] Almquist, H. J. 1941 Uncomplicated vitamin K deficiency in the rat. Federation Proc., *17*: 470 (abstract).

[7] Lecithin was obtained from Central Soya Company, Chicago.

[8] Technical glycerol trioleate was obtained from Drew Chemical Company, New York.

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Amino Acids in Gut Contents During Digestion in the Dog[1]

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Analyses of alimentary tract contents for free amino acids demonstrated that various test meals yielded similar amino acid mixtures in the small intestine (Nasset et al., '55). Gastric and duodenal contents arising from a test meal protein of unusual amino acid composition, such as zein, were usually identifiable. Jejunal and ileal contents could not be characterized in this way. The relative molar ratios of free amino acids in the part of the gut below the duodenum were relatively constant whether the test meal contained protein (egg albumin or zein) or not (lard or lard plus sucrose). These results were interpreted to mean that any meal, during digestion, is mixed with enough endogenous protein such as secreted enzymes and glycoproteins, as well as sloughed mucosa, to obscure the chemical identity of ingested exogenous protein. This interpretation was confirmed by feeding C^{14}-labeled casein. Dilution of this exogenous labeled protein with endogenous protein, in the gut, was at least fourfold in the dog and sixfold in the rat (Nasset and Ju, '61). The data referred to above were obtained mainly in acute experiments.

The experiments to be described here were designed to permit repeated observations on the same enterostomized, conscious animals, to determine the effects of amino acid solutions as test meals, and to compare, in a preliminary way, the microbiological and column chromatographic methods of amino acid analysis (Spackman, et al., '58).

METHODS

Jejunostomies were established about 50 cm caudad from the ligament of Treitz in two bitches of approximately 20-kg body weight. A catheter with terminal balloon was inserted a few centimeters downstream from the end-to-side anastomosis for the collection of gut contents. Samples were dehydrated in the frozen state. Lipids were removed and the free amino acids extracted from the fat-free solids with water, for microbiological determination, or with 1% aqueous picric acid, for chromatographic amino acid analysis. Details of analytical procedures are given elsewhere (Schreiber and Nasset, '59; Nasset and Ju, '61).

RESULTS

The micromoles of 18 amino acids in gut contents as determined by microbiological methods are listed in table 1. Experiments 1 and 2, as well as 3 and 4, were made on different days using lard only and meat only as the test meals. The duplicate experiments agree fairly well. The quantities of all amino acids are greater after meat feeding than after lard feeding but some of the differences are probably not significant.

In table 2 are listed the relative molar ratios of amino acids (threonine = 1.00) as determined from the mean values derived from table 1. These are listed under the column headed WARF. The same samples were analyzed later by the chromatographic method and the molar ratios derived from these results are in the column headed AAAA. The absolute values obtained by this method were always smaller than those obtained by the microbiological method. The differences were not uniform but varied from essentially identical results for aspartic acid to a 14-fold difference for tryptophan. The relative molar ratios of amino acids as observed in beef

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TABLE 1

Free amino acids recovered from a jejunostomy in dog 1 (micromoles/gram of dry, fat-extracted gut contents)[1]

Amino acids	Test meal			
	50 gm lard		50 gm lean beef	
	Experiment		Experiment	
	1	2	3	4
Alanine	31	29	74	73
Arginine	71	70	86	90
Aspartic	6.7	7.1	14	13
Cystine	4.2	3.7	4.6	12
Glutamic	76	67	184	159
Glycine	29	24	48	41
Histidine	8.6	5.5	15	13
Isoleucine	20	16	72	82
Leucine	57	38	72	82
Lysine	39	32	70	61
Methionine	9.4	11	12	16
Phenylalanine	20	14	25	31
Proline	12	10	15	16
Serine	48	42	62	63
Threonine	68	64	86	77
Tryptophan	15	13	20	21
Tyrosine	17	16	21	24
Valine	33	34	53	55

[1] Microbiological analyses performed by Wisconsin Alumni Research Foundation according to method of Henderson and Snell, J. Biol. Chem., 172: 15, 1948.

muscle protein are given for comparison in the last column of table 2.

Shown in table 3 are the relative molar ratios of the essential amino acids in gut contents after feeding various mixtures of pure amino acids in aqueous solution. These mixtures were made to duplicate the relative concentrations of the amino acids as observed in beef muscle and zein, and in gut contents after feeding lard. The total N in the amino acid test meals, 1.74 gm, was equal to that given in the 50-gm lean beef test meals. Microbiological amino acid analyses were used. These experiments were done to determine whether the digestive tract would alter the amino acid composition of its contents if given a mixture of amino acids previously demonstrated to arise from the feeding of protein or nonprotein test meals.

DISCUSSION

Judging by the data in table 1 an appreciable quantity of free amino acids is found in the small intestine even when no protein is ingested in the test meal. In experiments 1 and 2 the dry, fat-free con-

TABLE 2

Relative molar ratios in gut contents (dog 1, jejunostomy) by microbiological (WARF)[1] *and chromatographic (AAAA)*[2] *methods*[3] *and in beef muscle*[4]

Amino acids	Test meal				Beef muscle protein
	50 gm lard		50 gm lean beef		
	WARF	AAAA	WARF	AAAA	
Alanine	0.45	2.62	0.90	2.07	1.89
Arginine	1.07	1.64	1.08	1.93	0.98
Aspartic	0.15	1.13	0.17	1.07	1.56
Cystine	0.06	—	0.10	0.09	0.15
Glutamic	1.09	1.80	2.10	2.67	2.57
Glycine	0.41	3.77	0.55	1.60	1.72
Histidine	0.11	0.20	0.17	0.41	0.47
Isoleucine	0.27	0.98	0.94	1.07	0.93
Leucine	0.73	3.61	0.94	2.33	1.58
Lysine	0.55	2.46	0.80	2.60	1.52
Methionine	0.15	1.11	0.17	0.80	0.43
Phenylalanine	0.26	0.67	0.34	1.33	0.71
Proline	0.17	0.89	0.19	0.67	1.12
Serine	0.68	1.97	0.77	1.67	1.26
Threonine	1.00	1.00	1.00	1.00	1.00
Tryptophan	0.21	0.16	0.26	0.23	0.14
Tyrosine	0.26	1.03	0.28	1.20	0.44
Valine	0.52	1.10	0.66	1.07	1.21

[1] Microbiological analyses performed by Wisconsin Alumni Research Foundation according to method of Henderson and Snell, J. Biol. Chem., 172: 15, 1948.
[2] Spinco/Beckman Model 120 Automatic Amino Acid Analyzer.
[3] Each ratio is derived from the mean of two experiments.
[4] Computed from data in Amino Acid Handbook, eds., Block, R. J., and K. W. Weiss. 1956. Charles C Thomas, Springfield, Illinois, p. 270.

TABLE 3

Relative molar ratios of amino acids present in beef muscle and zein and in gut contents obtained from a jejunostomy after feeding amino acid mixtures (test meals given by stomach tube — microbiological amino acid analyses)

Amino acid	Beef muscle[1]	Test meal: AA = beef muscle[2]	Zein[1]	Test meal: AA = zein[3]	Gut contents after lard[4]	Test meal: AA = lard contents[5]
Arginine	0.98	0.93	0.41	0.67	1.79	1.52
Histidine	0.47	0.53	0.44	0.39	0.20	0.41
Isoleucine	0.93	0.73	2.21	1.06	0.55	0.62
Leucine	1.58	1.80	7.17	3.19	1.02	1.57
Lysine	1.52	1.31	0.00	0.62	0.68	1.21
Methionine	0.43	0.29	0.61	0.32	0.23	0.21
Phenylalanine	0.71	0.73	1.54	1.02	0.39	0.61
Threonine	1.00	1.00	1.00	1.00	1.00	1.00
Tryptophan	0.14	0.39	0.02	0.30	0.16	0.34
Valine	1.21	1.34	1.02	0.99	0.87	0.97

[1] Computed from data in Amino Acid Handbook, eds., Block, R. J., and K. W. Weiss, 1956. Charles C Thomas, Springfield, Illinois.
[2] Test meal was a solution of amino acids, in the proportions as found in beef muscle. Mean from two dogs.
[3] Test meal was a solution of amino acids, in the proportions as found in zein. Results from one dog.
[4] Gut contents after feeding 50 gm of lard as in table 1. Mean from two dogs.
[5] Test meal was a solution of amino acids, in the proportions as found in gut contents after feeding lard. See footnote 4. Mean from two dogs.

tents weighed 7.3 and 8.2 gm and contained 4.14 and 4.05% of total N, respectively. The sum of the mean values of the 18 free amino acids determined, microbiologically, is 68 mg of free amino acids per gm of dry, fat-free solids, making a total of 0.53 gm of amino acids, which constitutes 26% of the nitrogenous material present computed as protein ($N \times 6.25$). This represents the response to a non-nitrogenous test meal.

After feeding 1.74 gm of N in the form of lean beef (exp. 3 and 4, table 1) the mass of material recovered was greater and it contained more N. The dry, fat-free contents weighed 13.5 and 14.1 gm and contained 7.43 and 7.64% total N, respectively. The mean total mass of the 18 amino acids determined was 1.98 gm which is 30% of the total nitrogenous material present.

The amino acid composition of jejunal contents is not grossly altered by different types of test meals. In table 2 a comparison is possible of relative amino acid molar ratios in two gut contents, according to two analytical methods, and in beef muscle. The series of relative molar ratios of amino acids in gut contents after feeding lard or lean beef resemble each other much more closely than either one resembles the series obtained from the amino acids present in lean beef itself. The outstanding differences between lard and beef gut contents are in alanine, glutamic acid and isoleucine. The outstanding differences between beef contents and beef itself are in alanine, aspartic acid, glycine, histidine, lysine, methionine, phenylalanine, proline, tryptophan and valine. Very large differences are evident between the values for the microbiological (WARF) and the chromatographic (AAAA) methods. Comparison between beef gut contents, by the chromatographic method, and beef itself again reveals large discrepancies in about half of the amino acids concerned. These results indicate that it is difficult to change suddenly the qualitative make-up of the amino acid mixture in the gut. If a deficient diet were to be fed to the animal for some time prior to the test the response might be quite different. The animals used in these experiments regularly ate a normal diet and were simply fasted for 16 to 18 hours before being fed the test meals. The animals, therefore, were presumably in N equilibrium and were not suffering from any known dietary deficiency. It is important to bear these facts in mind when comparing our results with those of others obtained with animals that have subsisted for prolonged periods with diets deficient in one or more amino acids or other essential nutrients.

The reasons for the differences between the microbiological and the chromatographic methods for amino acids are unknown. The higher values obtained with the microbiological method suggest the presence in gut contents of non-amino acid growth-promoting substances. It is important to make a more detailed study of this problem. The molar ratios derived from use of the two methods are different. This difference does not invalidate our original interpretation, namely, that the amino acid mixture in the lumen of the intestine is not directly determined by the amino acid composition of the ingested protein.

SUMMARY

1. The relative molar ratios of free amino acids in the gut lumen were not greatly different whether protein or non-protein test meals were fed to enterostomized dogs.

2. Feeding test meals of aqueous amino acid solutions yielded gut contents with different proportions of amino acids.

3. The microbiological method yielded higher values than the column chromatographic method for amino acids.

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Utilization of Ethanol by Way of the Citric Acid Cycle in the Rat[1]

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A number of reports have appeared in the literature concerning the utilization of ethanol (Vitale et al., '53, '54; Westerfeld et al., '55, '59; Segovia-Riquelme et al., '56; Smith et al., '57; Quastel, '59). Whereas there is a unanimity of opinion that the oxidation of alcohol proceeds predominantly by way of the citric acid cycle, no definitive evidence has been offered as to the extent of the participation of the cycle in alcohol-treated subjects when compared to normals. More complete knowledge of the terminal sequence of reactions of alcohol oxidation, therefore, is needed. Because the liver is the main site for alcohol metabolism, a study of the cycle in this tissue obtained from ethanol-treated rats could provide invaluable information as to the activity of the cycle. Perhaps it may be added that, although the cycle components may be considered to serve a "catalytic" function, they are actually also intermediates in a chain of reactions and their levels may increase in tissues if an *excess* of utilizable substrate is present (Dajani and Orten, '58). The increase may be accentuated at appropriate stages of the cycle if inhibitors are also added (Dajani and Orten, '58). In the present investigation, isolated rat hepatic cells or homogenates were incubated with either acetate-1-C^{14} or ethanol-1-C^{14} in the presence or absence of inhibitors and the amounts and C^{14} activities of the citric acid cycle members were determined. The results clearly demonstrate that there are differences in the degree of utilization of the two substances in the livers of normal and alcohol-treated rats.

EXPERIMENTAL[2]

Procedure. Forty male Sprague-Dawley rats, weighing between 120 and 150 gm, were divided into two groups of comparable weights, 20 animals each. Both groups were maintained with the stock diet[3] for the entire period of study of 24 weeks. One group was given water and served as the control. The other group had access to 10% ethanol as the sole source of fluid. Both the diet and fluids were given ad libitum. After a three-week adjustment period the concentration of alcohol was raised to 20% and kept at this level until the termination of the experiment. The laboratory temperature was held at 76 to 78°F. and all possible cleanliness was maintained. Two rats from the alcohol-treated group and one from the control died during the study, and several animals died during the liver perfusion procedure described below.

Preparation of liver homogenates. At intervals of about 6 weeks after the preliminary adjustment period, two to three animals from each group were anesthetized with ether, the abdomens were opened and the livers were perfused *in situ* with ice-cold 0.9% NaCl solution until blanching was maximal. The livers were then removed, washed thoroughly with ice-cold saline from adhering blood and then pooled. Ten per cent homogenates of the pooled livers from each group were prepared in 50 ml of Krebs-Ringer-phosphate

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[2] Materials: DPN, ATP, L-malic acid, all purified grades, were purchased from the California Foundation for Biochemical Research, Los Angeles. Acetate-1-C^{14} and ethanol-1-C^{14} were obtained from Volk Biochemical Company, Chicago. Penicillin G potassium was obtained from Lederle Laboratories, New York, and dihydrostreptomycin from Chas. Pfizer and Company, New York. Highly purified monofluoroacetic acid was kindly supplied by Dr. J. J. Jasper, Wayne State University. Analytical reagent grades of all other chemicals were used.

[3] Rockland Farms Rat Pellets, Rockland Farms, New City (Rockland County), New York.

buffer (KRP), pH 7.4, at 4 to 5°C with the use of a Potter-Elvehjem glass homogenizer. To the homogenates were added the following substances, together with either ethanol or acetate, dissolved in 5 ml of KRP: (in milligrams) L-malic acid, 0.05; ATP, 8; DPN, 2; monofluoroacetic acid (where used), 0.5; sodium malonate (where used), 75; penicillin, 2 mg; streptomycin, 4. Sodium fluoride (0.01 M final concentration) was added to the KRP used in homogenization in order to minimize the possible loss of ATP and DPN by ATPase and DPNase, respectively. The amounts of substrates used are given as footnotes to the appropriate tables. The procedures for incubation, extraction, and chromatographic separation and determination of the acids followed those previously described (Dajani and Orten, '58).

Preparation of isolated hepatic cell suspensions. As with the homogenates, two to three rat livers were used for each preparation. The cells were isolated by the procedure of Branster et al. ('57), modified to include a hypertonic saline solution (2.7% sodium chloride), penicillin, 4 mg per 100 ml, and streptomycin, 8 mg per 100 ml in the perfusion mixture. An additional step was introduced into the original procedure, namely, washing the cells three times with 0.9% sodium chloride and then resuspending the free cells in 0.7% saline containing the antibiotics. The mixture was kept in the cold for 24 hours to equilibrate and for the cells to swell back to normal size. The cells were then centrifuged and 10% suspensions from each group were prepared in KRP not containing sodium fluoride. The remainder of the procedure followed that outlined above for homogenates. This modification gave a better yield of intact free cells than the original procedure and provided a more homogeneous suspension.

RESULTS

In the present study the utilization of acetate-1-C^{14} by way of the citric acid cycle was investigated first in homogenates made from livers obtained from normal and alcohol-treated rats immediately after the three-week preliminary adjustment period referred to above. No differences were observed at this stage of the experiment in the levels and C^{14} activities of the citric acid cycle intermediates isolated from the two groups. However, 6 weeks later and thereafter, marked differences were consistently noted in the amounts as well as the C^{14}-activities of the cycle acids in the two types of livers as is evident from the data presented in table 1. The results demonstrate clearly that acetate was better utilized by livers of the normal rats than by those of the alcohol-treated animals. With the exception of α-ketoglutarate, the quantities and activities of the cycle acids from the former group were always higher than the corresponding values from the latter.

The addition of the inhibitor, fluoroacetate, did not result in an accumulation of citrate in either group of rats (table 1). Indeed, there was some decrease in the amount of citrate as well as in those of the other cycle acids with the exception of the oxalacetate-acetoacetate fraction. On the other hand, the addition of malonate led to an increase in succinate, as might be expected. The increase in the alcohol-treated group, however, was less than that in the untreated animals, further supporting the observation that acetate is utilized to a lesser degree by the livers of the alcohol-treated rats than by those of the untreated animals.

In experiments employing liver homogenates and using ethanol as the substrate, however, the results obtained were different. Both the amounts and C^{14} activities of the Krebs cycle intermediates were less in livers of the control than in those of the alcohol-treated rats (table 2). As in the experiments with acetate, the levels of α-ketoglutaric acid were always higher than any other acid of the cycle isolated from the livers of the treated rats, of course, when blocking agents were not used.

Because results obtained with homogenates may be subject to alleged uncertainties due to cellular disorganization, the use of intact cells could offer a more suitable tissue preparation. Considering this, and because of the unexpected difference observed in the foregoing studies, the utilization of acetate and ethanol was reinvestigated in intact isolated hepatic cells. The results of these experiments are presented in tables 3 and 4. Whereas the cells from both types of livers exhibited higher

TABLE 1

Cycle acids[1] biosynthesized[2] per 100 gm of liver homogenates incubated with acetate-1-C^{14}

Acid	Acetate		Fluoroacetate added		Malonate added	
	μmoles	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$
			Untreated			
Citric	18.5 ± 0.7	9.9 ± 0.2	16.2 ± 0.4	8.3 ± 0.07	16.5 ± 0.5	8.4 ± 0.08
Aconitic (*cis* and *trans*)	5.6 ± 0.3	3.8 ± 0.1	1.2[3] ± 0.05	1.9 ± 0.02	2.3 ± 0.2	2.7 ± 0.03
Isocitric	5.0 ± 0.1	3.6 ± 0.08	1.1[3] ± 0.05	1.5 ± 0.03	2.2 ± 0.2	2.3 ± 0.02
α-Ketoglutaric	37.5 ± 0.6	8.6 ± 0.05	26.5 ± 0.35	6.5 ± 0.1	40.9 ± 0.3	9.1 ± 0.07
Succinic	40.0 ± 0.95	9.4 ± 0.15	39.0 ± 0.5	8.9 ± 0.2	58.0 ± 1.1	12.6 ± 0.1
Fumaric	36.0 ± 0.3	8.3 ± 0.1	34.3 ± 0.2	7.9 ± 0.05	31.0 ± 0.85	5.4 ± 0.04
Malic	30.0 ± 0.3	7.5 ± 0.1	29.0 ± 0.6	7.4 ± 0.03	22.0 ± 0.1	5.5 ± 0.05
Oxalacetic[4]	11.0 ± 0.2	6.1 ± 0.04	58.0 ± 0.8	29.6 ± 0.1	35.0 ± 0.8	19.5 ± 0.1
			Alcohol-treated			
Citric	14.0 ± 0.55	8.0 ± 0.1	13.0 ± 0.35	7.8 ± 0.05	13.3 ± 0.4	7.9 ± 0.07
Aconitic (*cis* and *trans*)	3.5 ± 0.2	2.5 ± 0.06	0.9[3] ± 0.4	1.6 ± 0.02	2.2 ± 0.2	2.3 ± 0.03
Isocitric	3.0 ± 0.2	2.4 ± 0.07	0.9[3] ± 0.05	1.6 ± 0.02	2.0 ± 0.2	2.0 ± 0.02
α-Ketoglutaric	41.0 ± 0.5	9.2 ± 0.1	30.4 ± 0.4	7.5 ± 0.08	42.7 ± 0.3	9.3 ± 0.06
Succinic	33.0 ± 0.65	7.8 ± 0.1	31.9 ± 0.6	7.7 ± 0.1	47.8 ± 0.9	10.8 ± 0.12
Fumaric	32.0 ± 0.15	7.7 ± 0.09	30.0 ± 0.3	7.5 ± 0.07	18.5 ± 0.95	5.0 ± 0.03
Malic	27.0 ± 0.3	6.6 ± 0.08	26.2 ± 0.7	6.5 ± 0.03	20.0 ± 0.15	5.2 ± 0.06
Oxalacetic[4]	9.5 ± 0.4	5.7 ± 0.05	51.6 ± 1.0	27.3 ± 0.09	29.0 ± 0.7	16.7 ± 0.15

[1] Average of 3 experiments, with average deviations, using homogenates prepared on the 10th, 16th and 22nd week of experiment, respectively.
[2] Values above control (substrate not added).
Substrates: NaAc·3H_2O, 3.5 mg; NaAc-1-C^{14} (activity = 2.5 mc/mmole), 1.0 mg; Malic acid, 0.05 mg; 10% suspension KRP, 50.0 ml; Monofluoroacetic acid (where used), 0.5 mg; and Na-malonate (where used), 75 mg.
[3] Amount too small to be measured accurately.
[4] Mixed with acetoacetate.

TABLE 2

Cycle acids[1] biosynthesized[2] per 100 gm of liver homogenates incubated with ethanol-1-C^{14}

Acid	Ethanol		Fluoroacetate added		Malonate added	
	μmoles	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$
			Untreated			
Citric	12.0 ± 0.5	20.5 ± 0.3	10.9 ± 0.3	18.7 ± 0.1	11.5 ± 0.4	19.4 ± 0.1
Aconitic (*cis* and *trans*)	5.5 ± 0.25	12.2 ± 0.2	1.1[3] ± 0.1	2.4 ± 0.05	2.4 ± 0.2	3.8 ± 0.05
Isocitric	5.0 ± 0.2	11.8 ± 0.1	1.0[3] ± 0.1	2.3 ± 0.04	2.3 ± 0.1	3.7 ± 0.03
α-Ketoglutaric	17.4 ± 1.0	17.8 ± 0.1	12.4 ± 0.8	12.8 ± 0.4	21.5 ± 1.3	22.3 ± 0.09
Succinic	17.9 ± 1.1	16.2 ± 0.2	17.6 ± 0.6	15.0 ± 0.2	27.5 ± 1.5	29.0 ± 0.1
Fumaric	17.6 ± 0.5	15.4 ± 0.1	17.0 ± 0.5	14.8 ± 0.2	11.9 ± 0.8	10.5 ± 0.08
Malic	17.0 ± 1.2	14.9 ± 0.09	16.7 ± 0.7	14.7 ± 0.1	11.6 ± 0.3	10.2 ± 0.1
Oxalacetic[4]	6.0 ± 0.8	8.9 ± 0.05	33.0 ± 1.4	41.2 ± 0.15	20.8 ± 0.9	25.0 ± 0.07
			Alcohol-treated			
Citric	20.0 ± 0.7	33.5 ± 0.6	17.8 ± 0.7	30.7 ± 0.2	18.6 ± 1.0	32.6 ± 0.15
Aconitic (*cis* and *trans*)	7.0 ± 0.3	15.4 ± 0.3	1.2[3] ± 0.1	2.5 ± 0.03	2.6 ± 0.15	4.0 ± 0.04
Isocitric	6.3 ± 0.2	14.3 ± 0.2	1.1[3] ± 0.15	2.4 ± 0.04	2.5 ± 0.1	3.9 ± 0.04
α-Ketoglutaric	37.5 ± 1.3	38.2 ± 0.15	26.0 ± 1.1	27.0 ± 0.3	39.8 ± 1.2	41.1 ± 0.1
Succinic	30.0 ± 0.9	26.0 ± 0.25	24.6 ± 0.9	21.9 ± 0.1	42.5 ± 1.7	40.0 ± 0.15
Fumaric	28.0 ± 0.7	24.3 ± 0.1	23.7 ± 0.6	20.5 ± 0.3	21.4 ± 1.0	18.7 ± 0.09
Malic	27.5 ± 1.0	23.5 ± 0.08	22.0 ± 0.8	19.8 ± 0.2	21.3 ± 0.4	18.4 ± 0.06
Oxalacetic[4]	12.0 ± 0.7	17.0 ± 0.07	52.0 ± 1.2	64.8 ± 0.4	35.2 ± 1.1	45.3 ± 0.18

[1] Average of 3 experiments, with average deviations, using homogenates prepared on the 10th, 16th and 22nd week of experiment, respectively.
[2] Values above control level (no substrate added).
Substrates: EtOH, 95%, 2.0 ml; EtOH-1-C^{14}, 0.2 mc; Malic acid, 0.05 mg; 10% suspension in KRP, 50 ml.
[3] Amount too small to be determined accurately.
[4] Mixed with acetoacetate.

activity of the cycle than homogenates, thus supporting the rationale of using isolated cells, again acetate was better utilized and ethanol less well metabolized by the control than by the alcohol-treated livers. As before, α-ketoglutarate was the one exception, being higher in the alcohol-treated liver cells.

Along with the cycle intermediates, pyruvic and lactic acids were also determined in every experiment. Although the amounts of pyruvic and lactic acids in both groups were not markedly different, they were consistently higher in the alcohol-treated group. When alcohol was used as a substrate, the difference was even more evident (table 5).

DISCUSSION

It was demonstrated in the present experiments with acetate (tables 1 and 3) that the livers of alcohol-treated animals utilized less acetate in the acids of the Krebs cycle than the controls. On the other hand, the tissue preparations from the treated rats utilized more ethanol (tables 2 and 4). This may seem paradoxical especially if one assumes that acetate is an obligatory intermediate in the reaction of ethanol oxidation (Leloir and Munez, '38; Wahlenstein and Weinhouse, '53). Although it is not yet clear why alcohol-treated livers react in this manner, a partial explanation may be proposed. It could be that the levels and/or turnover of one or more of the enzymes in the sequence of the reactions from ethanol to acetaldehyde to acetyl CoA, with a by-passing of acetate, is increased. This could account for the better utilization of ethanol via the citric acid cycle. Observations on this point will be reported in a subsequent communication. An alternative mechanism would be an alteration in some of the reactions concerned with the conversion of acetate to acetyl CoA with the resultant slowing of the acetate flux into the oxidative cycle in the alcohol-treated rats, but apparently not involving the acylating enzymes, as the latter are required for acetyl CoA formation from both acetate and ethanol. This speculation would be justified, of course, if acetate is not an obligatory intermediate but a product of a side reaction in ethanol oxidation. Indeed, free acetic acid was shown by Himwich et al. ('33) as well as by Friedemann and associates ('39), not to accumulate during alcohol metabolism *in vivo*. These authors interpreted this to mean that acetate either was not formed directly or it was further metabolized as rapidly as it was formed. Such a conclusion would likewise appear to be reasonable in the present investigation if one compares the data from the alcohol-treated group with that of the untreated group. The picture is perhaps less clear, however, if one compares the utilization of acetate by the alcohol-treated group (tables 1 and 3) with the utilization of alcohol by these groups (tables 2 and 4). The 6-C-acids as well as the oxalacetate-acetoacetate fractions are consistently higher when ethanol is the substrate. The C-5 and other C-4 acids are somewhat higher when acetate is the substrate. Several explanations for this observation may be suggested. Differences in substrate concentrations may, of course, be a factor. Or perhaps some usually minor alternate pathway(s) of acetate entrance into the cycle may be operative, e.g., acetate to succinate by tail-to-tail condensation (Dajani and Orten, '58; Davis, '58). Or perhaps the higher levels of C-4 acids may be simply a reflection of the higher level of α-ketoglutarate when acetate is used as a substrate.

The possibility of a direct conversion of acetaldehyde to acetyl CoA in liver by means of acetaldehyde dehydrogenase (Black and Hutchens, '48; Burton and Stadtman, '53), glyceraldehyde phosphate dehydrogenase (Lipmann, '45; Racker and Krimsky, '52), or some unknown enzyme is enhanced by the observation that acetaldehyde is a better source of fatty acids than acetate (Brady and Gurin, '51). In view of the present data it would not be unreasonable to speculate, therefore, that acetate, if formed, arises from the deacylation of the acetyl-CoA biosynthesized directly from ethanol. If this is the case it would not be unexpected to find, as was observed in this study, that the two types of livers under consideration utilize acetate and ethanol to a different extent.

Another interpretation of the higher values and C^{14} activities for the cycle acids in the untreated animals, when acetate was used as the substrate, could be that the exit

TABLE 3

Cycle acids[1] biosynthesized[2] per 100 gm of isolated liver cells incubated with acetate-1-C^{14}

Acid	Acetate		Fluoroacetate added		Malonate added	
	μmoles	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$
			Untreated			
Citric	19.5 ± 0.6	10.2 ± 0.18	17.8 ± 0.3	9.1 ± 0.08	18.2 ± 0.37	9.4 ± 0.06
Aconitic (*cis* and *trans*)	5.7 ± 0.3	3.9 ± 0.1	1.3[3] ± 0.07	2.0 ± 0.03	2.4 ± 0.2	2.8 ± 0.02
Isocitric	5.0 ± 0.15	3.6 ± 0.09	1.2[3] ± 0.05	1.7 ± 0.03	2.3 ± 0.15	2.5 ± 0.02
α-Ketoglutaric	42.0 ± 0.5	9.4 ± 0.06	28.7 ± 0.4	7.0 ± 0.09	44.6 ± 0.3	10.0 ± 0.08
Succinic	43.0 ± 1.0	10.9 ± 0.15	40.0 ± 0.5	9.7 ± 0.17	63.5 ± 1.0	13.0 ± 0.09
Fumaric	38.5 ± 0.4	9.2 ± 0.1	36.5 ± 0.25	8.5 ± 0.07	24.0 ± 0.7	5.9 ± 0.05
Malic	33.5 ± 0.35	8.0 ± 0.1	31.4 ± 0.4	7.6 ± 0.04	25.7 ± 0.15	6.1 ± 0.05
Oxalacetic[4]	12.5 ± 0.3	6.7 ± 0.05	62.5 ± 1.2	32.0 ± 0.1	38.4 ± 0.6	21.0 ± 0.08
			Alcohol-treated			
Citric	15.5 ± 0.5	8.3 ± 0.15	13.3 ± 0.25	7.9 ± 0.07	13.7 ± 0.4	8.3 ± 0.07
Aconitic (*cis* and *trans*)	3.8 ± 0.25	2.8 ± 0.07	1.1[3] ± 0.05	1.8 ± 0.04	2.3 ± 0.15	2.4 ± 0.03
Isocitric	3.2 ± 0.2	2.5 ± 0.06	1.0[3] ± 0.04	1.7 ± 0.02	2.2 ± 0.16	2.2 ± 0.02
α-Ketoglutaric	45.5 ± 0.4	9.9 ± 0.08	31.6 ± 0.35	7.7 ± 0.06	47.5 ± 0.35	10.4 ± 0.05
Succinic	35.5 ± 0.75	8.8 ± 0.1	33.0 ± 0.7	7.9 ± 0.1	50.9 ± 0.75	11.2 ± 0.09
Fumaric	35.0 ± 0.3	8.4 ± 0.1	31.5 ± 0.67	7.6 ± 0.08	19.8 ± 0.6	5.5 ± 0.04
Malic	29.5 ± 0.2	7.2 ± 0.1	27.8 ± 0.3	6.8 ± 0.03	21.5 ± 0.2	5.7 ± 0.05
Oxalacetic[4]	10.0 ± 0.15	5.9 ± 0.04	53.0 ± 0.8	28.5 ± 0.08	31.4 ± 0.7	18.0 ± 0.1

[1] Average of 3 experiments, with average deviations, using isolated cell preparations made on the 12th, 18th, and 24th week of experiment, respectively.
[2] Values above control (substrate not added).
Substrates: NaAc·3H$_2$O, 3.5 mg; NaAc-1-C^{14} (activity = 2.5 mc/mmole), 1.0 mg; Malic acid, 0.05 mg; 10% suspension KRP, 50.0 ml; Monofluoroacetic acid (where used), 0.5 mg; Na-malonate (where used), 75.0 mg.
[3] Amount too small to be measured accurately.
[4] Mixed with acetoacetate.

TABLE 4

Cycle acids[1] biosynthesized[2] per 100 gm of isolated liver cells incubated with ethanol-1-C^{14}

Acid	Ethanol		Fluoroacetate added		Malonate added	
	μmoles	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$
			Untreated			
Citric	12.9 ± 0.4	21.7 ± 0.2	11.8 ± 0.25	20.0 ± 0.15	12.4 ± 0.4	20.5 ± 0.1
Aconitic (*cis* and *trans*)	5.7 ± 0.2	12.3 ± 0.15	1.3[3] ± 0.1	2.5 ± 0.08	2.5 ± 0.17	4.0 ± 0.06
Isocitric	5.2 ± 0.2	12.0 ± 0.1	1.2[3] ± 0.1	2.4 ± 0.05	2.4 ± 0.1	3.9 ± 0.04
α-Ketoglutaric	18.5 ± 1.0	18.2 ± 0.15	13.1 ± 0.7	13.5 ± 0.3	23.0 ± 1.1	24.2 ± 0.1
Succinic	18.8 ± 1.0	17.3 ± 0.1	18.5 ± 0.5	15.7 ± 0.19	28.8 ± 1.3	30.5 ± 0.2
Fumaric	18.3 ± 0.5	16.0 ± 0.1	17.7 ± 0.6	15.4 ± 0.19	12.5 ± 0.8	11.0 ± 0.09
Malic	17.9 ± 1.0	15.8 ± 0.1	17.6 ± 0.6	15.3 ± 0.1	12.4 ± 0.4	10.9 ± 0.15
Oxalacetic[4]	7.0 ± 0.7	9.5 ± 0.04	38.5 ± 1.3	48.0 ± 0.2	24.3 ± 0.8	39.2 ± 0.07
			Alcohol-treated			
Citric	21.5 ± 0.6	34.8 ± 0.6	19.3 ± 0.8	33.0 ± 0.15	20.0 ± 0.2	35.0 ± 0.1
Aconitic (*cis* and *trans*)	7.3 ± 0.25	16.0 ± 0.2	1.3 ± 0.15	2.6 ± 0.04	2.7 ± 0.2	4.2 ± 0.05
Isocitric	6.6 ± 0.3	15.3 ± 0.16	1.2 ± 0.1	2.5 ± 0.03	2.6 ± 0.1	4.1 ± 0.04
α-Ketoglutaric	39.8 ± 1.2	39.2 ± 0.1	27.0 ± 1.2	28.0 ± 0.2	40.5 ± 1.0	42.5 ± 0.15
Succinic	31.2 ± 1.0	27.5 ± 0.2	25.5 ± 1.1	22.8 ± 0.1	44.3 ± 1.5	41.6 ± 0.14
Fumaric	29.1 ± 0.8	25.4 ± 0.1	24.6 ± 0.7	21.4 ± 0.3	22.3 ± 1.0	19.4 ± 0.1
Malic	28.9 ± 1.0	25.3 ± 0.09	24.0 ± 0.9	21.6 ± 0.2	23.2 ± 0.3	19.8 ± 0.05
Oxalacetic[4]	14.0 ± 0.7	18.5 ± 0.06	61.0 ± 1.0	75.2 ± 0.3	41.0 ± 1.0	52.7 ± 0.2

[1] Average of 3 experiments, with average deviations, using isolated cell preparations made on the 12th, 18th, and 24th week of experiment, respectively.
[2] Values above control level (no substrate added).
Substrates: EtOH, 95%, 2.0 ml; EtOH-1-C^{14}, 0.2 mc; Malic acid, 0.05 mg; 10% suspension in KRP, 50 ml.
[3] Amount too small to be measured accurately.
[4] Mixed with acetoacetate.

TABLE 5

Amounts[1] and C^{14} activities of pyruvic and lactic acids biosynthesized per 100 gm of tissue preparation

Acid	Acetate				Ethanol			
	Untreated		Alcohol-treated		Untreated		Alcohol-treated	
	μmoles	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$	*μmoles*	*count/min.* $\times 10^{-3}$
				Isolated liver cells				
Pyruvic	14.0 ± 0.8	3.0 ± 0.05	16.8 ± 0.9	3.5 ± 0.04	28.0 ± 1.2	15.3 ± 0.1	40.0 ± 1.0	27.8 ± 0.15
Lactic	30.0 ± 1.0	5.6 ± 0.07	33.0 ± 0.4	5.7 ± 0.1	32.0 ± 1.2	17.6 ± 0.09	68.0 ± 2.0	32.6 ± 0.18
				Liver homogenates				
Pyruvic	12.5 ± 0.7	2.8 ± 0.05	15.0 ± 0.6	3.2 ± 0.07	25.9 ± 0.9	14.0 ± 0.15	37.0 ± 0.8	25.0 ± 0.2
Lactic	28.0 ± 0.8	5.1 ± 0.03	30.5 ± 0.5	5.2 ± 0.02	30.0 ± 1.0	16.0 ± 0.1	62.0 ± 1.1	30.2 ± 0.2

[1] Average of 3 experiments with average deviation, using homogenates or isolated liver cell preparations made on the 10th, 16th, 22nd, and on the 12th, 18th and 24th week of experiment, respectively.

mechanisms of the acids from the cycle are less active, thus producing a build-up of the intermediates. A direct answer to this rather involved question is, of course, not possible from the present data. However, other studies (Dajani and Orten, '58) have shown a direct relationship between the "metabolic activity" of different tissues and the levels and C^{14} activities of the citric acid cycle acids. For example, the amounts and C^{14} activities of the cycle acids, using C^{14}-labeled acetate as a substrate, were far higher in nucleated than in nonnucleated erythrocytes. The difference was accentuated by the use of fluoroacetate or malonate as blocking agents. The same relationship held in liver, muscle and certain tumor tissues (Dajani et al., '61).

Still other explanations of the differences in the levels of the cycle acids and their C^{14} activities with different substrates could be suggested. These include the questions of relative rates of transfer of acetate and ethanol across the cell and mitochondrial membranes, and the possible effects of lowered food (and protein) intakes in the alcohol-fed rats on the enzyme activities by dilution or concentration of mitochondrial as opposed to soluble cytoplasmic enzymes. These, as well as other possible interpretations, obviously require further investigation for their elucidation.

The question of the consistently elevated α-ketoglutaric acid in the livers when either acetate or ethanol is used as a substrate (columns 1 and 7, tables 1–4) requires further comment. Several mechanisms could contribute to this finding. Perhaps the most reasonable is an increased activity of some by-pass mechanism leading directly to the formation of α-ketoglutarate, such as the "succinate-glycine cycle" (Shemin et al., '55). Indeed, the increased excretion of coproporphyrin formed presumably by way of this cycle, in human alcoholics (Sutherland and Watson, '51), supports such a view. Another possibility is that an alteration occurs in some other enzymatic reactions which lead to or follow α-ketoglutarate formation. Although it is not possible at present to assess the exact nature of these changes, it might be logical to suggest that one or more of the enzymes involved may be more sensitive to alcohol habituation than the others, and that they

are the first to be affected by alcohol treatment. On this basis, one might predict a more complete reduction in the remaining steps of the cycle after prolonged treatment with ethanol. These points merit further investigation.

As stated earlier, the addition of fluoroacetate to the liver preparations did not block the cycle completely, leading to an increase in the oxalacetate-acetoacetate fraction rather than to an accumulation of citrate. As observed in the present investigation, other workers (Potter and Busch, '50) reported that liver is unique in that fluoroacetate inhibition does not produce an accumulation of citrate as occurs in most other tissues but instead causes a diversion of acetate into fatty acid synthesis with a resulting increased formation of acetoacetate. A similar rise in the acetoacetate level was noticed in our earlier studies on nucleated erythrocytes (Dajani and Orten, '58) under comparable experimental conditions. Furthermore, in this investigation higher concentrations of fluoroacetate were observed to increase blocking substantially in the entire cycle. Obviously, this was undesirable in the present study.

The higher levels of pyruvate and lactate (table 5) noted in treated livers when ethanol was used as a substrate (table 2) can be explained by the premise that more pyruvate is formed from the increased amounts of oxalacetate through decarboxylation reactions (Wood and Werkman, '36; Evans et al., '43) or from malic acid by the malic enzyme system (Ochoa et al., '48; Ochoa, '51; Harary et al., '53). A part of this larger quantity of pyruvate, in turn, would be reduced rapidly to lactate by the lactic dehydrogenase system (Meyerhof and Lohman, '26) especially if DPNH is present in sufficiently large amounts. Greater quantities of DPNH would be formed, of course, during ethanol oxidation and more so, to be sure, if the levels of both alcohol and acetaldehyde dehydrogenases were elevated in the alcohol-treated livers. This reasoning is in accord with the observation of Smith and Newman ('59) who showed that the DPN/DPNH ratios were lowered during active alcohol metabolism in the intact animal. As a matter of fact, the data in table 5 on lactate support an earlier observation by Eggleton ('46) that acidosis following alcohol ingestion is not due to acetic acid but is related to the increased blood lactate, presumably arising from the reduction of pyruvate.

Whereas no attempt was made to conduct histologic examinations on livers at any stage of the study, macroscopic observations were always recorded. The appearance and size of the livers from only the alcohol-treated rats were suggestive of fatty infiltration. Indeed, more fat-like globules from these livers in contrast with normal livers were seen microscopically during the preparation of isolated liver cells. This is not unexpected in animals because it is believed that fatty livers develop in chronic alcoholics due to a very poor diet in which the main source of calories consists of alcohol (Chaikoff et al., '48; Williams, '59). It is also possible that alcohol acts like dietary fat in increasing the choline requirement (Forbes and Duncan, '50; Klatskin et al., '54), or if present in relatively large concentrations it leads to an accumulation of acetyl CoA in the liver. The latter leads to lipid synthesis by condensation and subsequent reduction due to the increase DPNH/DPN ratio (Quastel, '59).

SUMMARY

An *in vitro* comparative study was made of the utilization of acetate-1-C^{14} and ethanol-1-C^{14} by way of the citric acid cycle in liver homogenates and isolated liver cells from ethanol-treated and untreated rats. The results demonstrated that acetate is utilized more readily by the livers of the normal rats than by those of ethanol-treated animals. In contrast, ethanol is better utilized by the livers of the treated rats.

Possible relationships of these differences to the activities of the enzyme systems involved in ethanol oxidation were discussed.

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Effect of Dietary Protein Level on Growth and Liver Enzyme Activities of Rats

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There are many reports showing that the activities of various enzymes in animal tissues are affected by nutritional alterations. A number of workers have investigated the effect of a protein-free or low protein diet upon the activities of liver enzymes in animals, as compared with that of a diet containing an adequate amount of protein. In general, liver enzymes were labile, and different enzymes responded to protein depletion in different ways (Miller, '48, '50; Litwack et al., '52; Rosenthal et al., '52; Wainio et al., '53, '59; Zigman and Allison, '59; Sobrón and Sánchez, '61; Allison, '55; Knox et al., '56). There have been few studies, however, on the changes in liver enzyme activities of animals receiving diets containing different levels of protein. Lightbody and Kleinman ('39) and Mandelstam and Yudkin ('52) found that the activity of liver arginase in rats increased with an increase in dietary protein content. Recently, a similar response in liver glutamic-pyruvic transaminase activity of rats was also reported by Rosen et al. ('59). Ross and Batt ('56) indicated that the activity of liver alkaline phosphatase in rats increased when they were fed either low or high protein diets. Litwack and associates ('52, '53, '54) reported the effects of the quantity and quality of dietary protein on the activity of liver xanthine oxidase in rats. These investigators described a method using the response in liver xanthine oxidase activity as a criterion of protein quality. The present authors reported that the activities of liver xanthine oxidase and arginase in rats were affected but in different ways by dietary protein levels (Muramatsu and Ashida, '55, '57).

The present study was planned to provide additional data on the response of several liver enzymes in rats to various protein levels, and to examine whether the response is related to the growth rate.

The enzymes selected were xanthine oxidase, uricase, arginase, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, cathepsin, D-amino acid oxidase, glutamic dehydrogenase, succinic dehydrogenase and alkaline phosphatase.

EXPERIMENTAL

Animals and diets. Male weanling rats of the Wistar strain were used. All rats were fed the basal diet containing 25% of casein for 4 to 5 days before being offered the experimental diets, to compensate for any lack of uniformity in previous dietary history. Rats weighing 55 to 60 gm were separated into groups and fed the experimental diets ad libitum. The diets contained zero to 60% of casein (5 to 8 levels of casein were used), 85 to 25% of α-potato starch, 5% of sesame oil, 4% of Salts 6 (Hegsted et al., '41), 5% of cellulose powder, and 1% of vitamin mixture (Muramatsu and Ashida, '61). Variations in the protein content were made at the expense of starch. Each rat received two drops of cod liver oil per week as the source of fat-soluble vitamins. Five animals per group were used, except that 10 animals were used in the protein-free group. The animals were weighed every day.

Assay procedure. Rats were killed two or 4 weeks after being given the experimental diets. The liver was removed as rapidly as possible, wiped free of blood by mildly pressing with filter papers, weighed, and homogenized with 5 volumes of cold distilled water in a Waring Blendor fitted

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with an ice-jacket. The homogenate was filtered through two layers of gauze and an aliquot of the filtrate was used for the assays of the enzymes. The liver homogenates of each two rats fed the protein-free diet were pooled.

Xanthine oxidase was measured by a modification (Muramatsu and Ashida, '61) of the Dhungat and Sreenivasan ('54) method. The activity of this enzyme was expressed as microliters of oxygen consumed per hour per gram of wet liver. *Uricase* was measured by the same method except that 1.0 ml of the homogenate was diluted two times with distilled water and 1.0 ml of 0.3% lithium urate was used as substrate. After equilibration, the substrate was tipped and a reading was made after 20 minutes. The activity of uricase was expressed as microliters of oxygen consumed per 20 minutes per gram of wet liver. D-*Amino acid oxidase* was measured as follows: the main compartment of a Warburg flask contained 1.0 ml of the homogenate, 1.0 ml of pyrophosphate buffer, pH 8.3. The side arm contained 1.0 ml of 2.0% DL-alanine, and the center well contained 0.2 ml of 20% KOH. After incubation for 10 minutes at 37°C, the substrate was tipped, and manometric readings were taken every 20 minutes for 60 minutes. The activity of D-amino acid oxidase was expressed as microliters of oxygen consumed per hour per gram of wet liver. *Arginase* was measured by our method previously reported (Muramatsu and Ashida, '55), and the activity of this enzyme was expressed as milligrams of urea formed per hour per gram of wet liver. *Glutamic-pyruvic transaminase* and *glutamic - oxaloacetic transaminase* were measured by the methods of Caldwell and McHenry ('53) and Tonhazy et al. ('50), respectively. In the case of glutamic-oxaloacetic transaminase, the oxaloacetic acid formed by the reaction was converted to pyruvic acid by anilin citrate. The activity of these enzymes was expressed as milligrams of pyruvic acid formed per 10 minutes per gram of wet liver. *Cathepsin* was measured by using the method of Anson ('38); 1.0 ml of the homogenate was added to the tube containing 3.0 ml of 2% beef hemoglobin and 1.0 ml of acetate buffer, pH 3.6. The mixture was incubated at 37°C for 20 minutes, and the reaction was stopped by adding 5.0 ml of 5% trichloroacetic acid. The aromatic product was determined on the centrifuged solution by means of the Folin-Ciocalteu reagent, with tyrosine as the standard. The activity of cathepsin was expressed as milligrams of tyrosine liberated per 20 minutes per gram of wet liver. *Succinic dehydrogenase* and *glutamic dehydrogenase* were measured as previously reported (Muramatsu and Ashida, '61) by a modification of the method of Sreenivasammurthy and Swaminathan ('54). The activity of these enzymes was expressed as milligrams of formazan formed per 30 minutes per gram of wet liver. *Alkaline phosphatase* was measured by using the method of Rosenthal et al. ('52); 0.8 ml of the homogenate was added to the tube containing 0.4 ml of 0.1 M veronal buffer, pH 9.2, 0.4 ml of 0.1 M sodium-β-glycerophosphate, 0.4 ml of 0.03 M magnesium chloride. After the mixture was incubated at 37°C for 60 minutes, 2.0 ml of 5% trichloroacetic acid were added, and the inorganic phosphate was estimated on an aliquot of the filtrate. The activity of alkaline phosphatase was expressed as milligrams of phosphorus liberated per hour per gram of wet liver. The assays of all enzymes above mentioned were carried out so that the linearity of enzyme activity versus amount of homogenate was obtained. *Liver nitrogen* was measured by the semimicro-Kjeldahl method.

RESULTS

The changes in body weight of rats fed diets containing various levels of casein for 15 days are shown in figure 1. In this figure, the absolute amounts of liver weight, and liver nitrogen per milligram of tissue of rats fed for 15 days are also shown. The growth rate increased as the level of casein increased from zero to 25% in the diet. The maximal growth was obtained by feeding the 25% casein diet, and the growth rate was roughly maintained for all groups receiving 25% or more of casein in the diet. The 60% casein diet, however, produced a slightly lower weight gain. The feeding of protein below 25% in the diet resulted in both decreased liver weight and liver nitrogen.

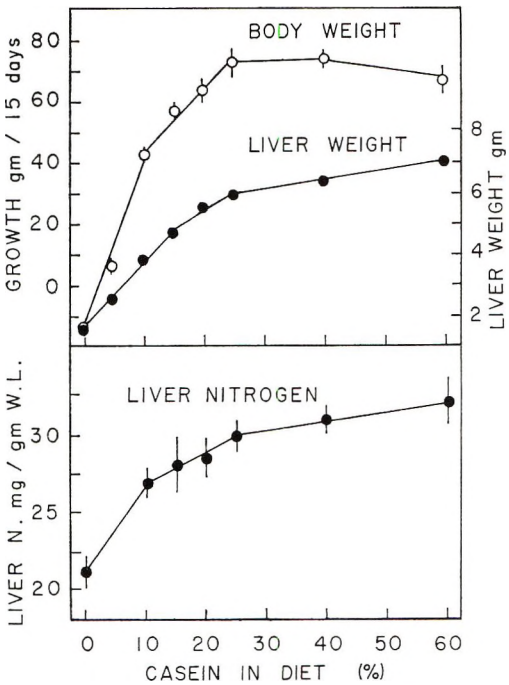

Fig. 1 Changes in body weight, liver weight and liver nitrogen of growing rats fed different levels of protein for 15 days. Vertical lines represent standard error of the mean.

The liver nitrogen content in animals maintained with a protein-free and the 10% casein diet was 35 and 15% lower, respectively, than that in rats fed the 25% casein diet. The values for the groups receiving 25% or more of casein in their diets did not differ from each other, although there was a tendency for them to increase with an increasing level of protein. The response curves of body weight, liver weight and liver nitrogen in rats fed diets containing different levels of protein for 29 days were roughly similar to those of the animals fed for 15 days.

The effects of feeding various levels of casein on the activities (per gram of tissue) of liver enzymes are shown in figures 2 to 6. The activity of liver xanthine oxidase (fig. 2) resulted in a marked decrease approaching zero in rats fed a protein-free diet; with increasing levels of casein, there was a linear increase in activity up to 25% of casein, with no further increase above that. These results are in agreement with our previous studies (Muramatsu et al., '54; Muramatsu and Ashida, '55, '57). It has also been reported by several workers that the activity of liver xanthine oxidase dropped markedly in rats receiving protein-free or low protein diets, and was one of the extremely labile enzymes in protein depletion (MacQuarrie and Venosa, '45; Miller, '48, '50; Westerfeld and Richert, '49; Williams and Elvehjem, '49; Litwack et al., '50; Wainio et al., '53).

The changes in the activity of liver succinic dehydrogenase (fig. 2) and glutamic dehydrogenase activities (fig. 3) of rats fed diets containing different protein levels were almost the same as those of liver xanthine oxidase. That is, the activities of these enzymes were increased with each increase in the casein content of the diet from zero to 25%, but did not greatly change with the 25% or more casein diets. The feeding of a protein-free or 10% casein diet, resulted in decreases of 90 and 57% in the activity of succinic dehydrogenase, and for both diets about 90% in that of glutamic dehydrogenase, respec-

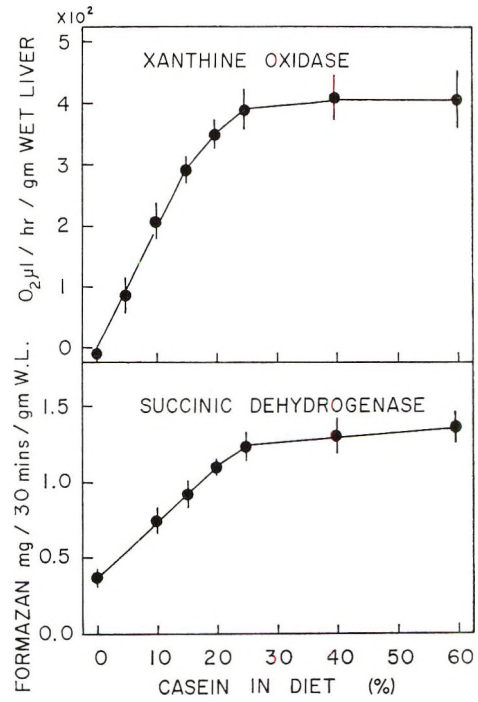

Fig. 2 Effect of different levels of protein on the activities of liver xanthine oxidase and succinic dehydrogenase in rats (15 days' feeding).

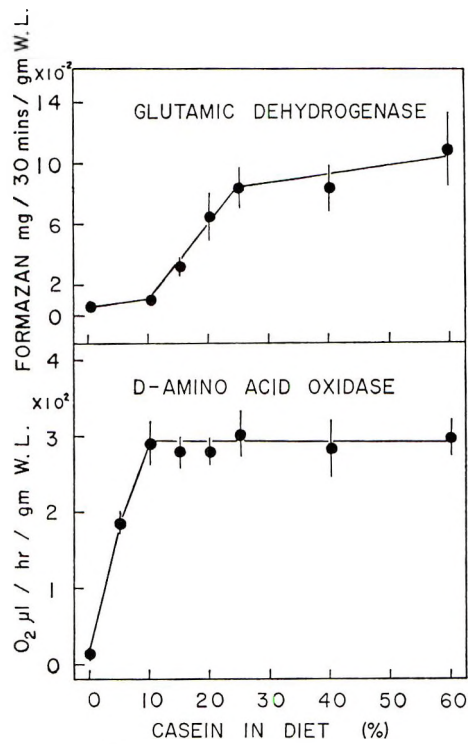

Fig. 3 Effect of different levels of protein on the activities of liver glutamic dehydrogenase and D-amino acid oxidase in rats (15 days' feeding).

tively, as compared with the values for rats receiving 25% casein diet.

The change in the activity of liver D-amino acid oxidase of rats fed diets containing various protein levels was also similar to that of liver xanthine oxidase, except that this activity attained the maximal activity in the rats fed the 10% casein diet (fig. 3). The activities of this enzyme in rats fed a protein-free and 5% casein diet were about 65 and 35% of the maximal value, respectively. Succinic dehydrogenase and D-amino acid oxidase have also been observed to decrease in protein-depleted rats (see Allison, '55; Knox et al., '56).

The activity of liver arginase (fig. 4) increased with a slight increase in casein content of the diet from zero to 25% (increased 1.3-fold), but increased significantly from 25 to 60%. The liver arginase activity of rats fed the 60% casein diet was fourfold that of the rats fed a protein-free diet. The response of the liver arginase activity agreed with our previous studies (Muramatsu and Ashida, '55, '57). Mandelstam and Yudkin ('52) indicated that liver arginase activity in rats increased proportionately with an increase in the level of casein from 20 to 60% in the diet.

The activity of liver uricase was shown to increase proportionally with the casein content of the diet (fig. 4). The activities of this enzyme in the liver of rats fed 10, 25 and 60% casein diets were 1.2-, 1.7- and 2.5-fold, respectively, that of rats fed a protein-free diet. Williams et al. ('50) reported that the uricase activity decreased in the livers of rats fed a low protein diet.

Similar responses were observed in liver glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase activities (fig. 5). The response curve obtained for the activity of liver glutamic-pyruvic transaminase resembles that reported by Rosen et al. ('59). The activities of liver glutamic-pyruvic transaminase in rats fed diets containing 10, 25, 40 and 60% casein

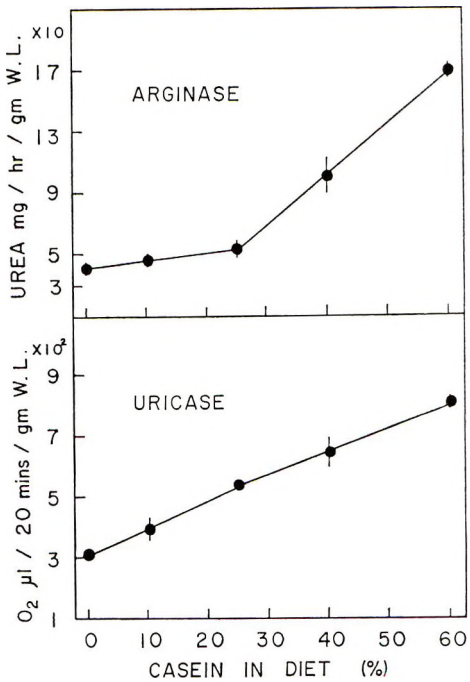

Fig. 4 Effect of different levels of protein on the activities of liver arginase and uricase in rats (15 days' feeding).

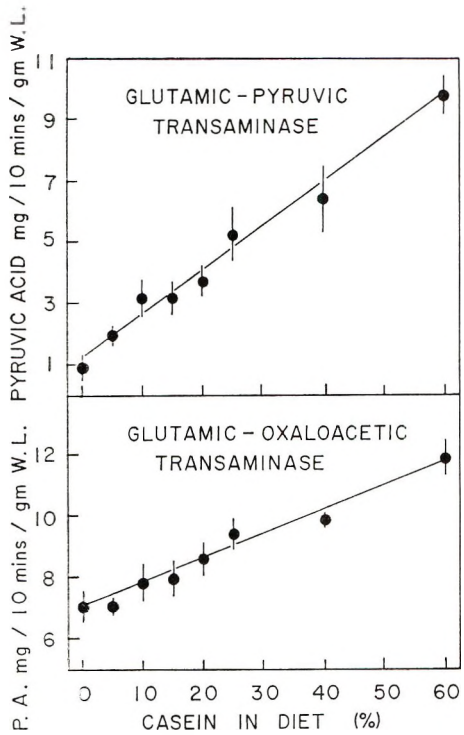

Fig. 5 Effect of different levels of protein on the activities of liver glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase in rats (15 days' feeding).

were 4.1-, 6.7-, 8.0- and 12.5-fold that of those fed a protein-free diet, respectively. In contrast, the increases in the activity of glutamic-oxaloacetic transaminase with their casein levels were only from 1.1- to 1.7-fold. It has been suggested that the increase in glutamic-pyruvic transaminase activity when rats were fed high protein was associated with increase in gluconeogenesis (Rosen et al., '59).

The response curves of the activity in any liver enzymes mentioned above did not significantly change even when the rats were fed for 15 or 29 days. Accordingly, only the results of rats fed for 15 days are described in this paper. On the other hand, the response curves of the activities of liver cathepsin and alkaline phosphatase showed a difference between rats fed for 15 and 29 days (fig. 6).

The activity of liver cathepsin decreased only in rats maintained with a protein-free diet for 15 days (a decrease of 25% from the maximal value), but did not noticeably change in the other groups (fig. 6). Data of Miller ('48, '50) showed that the activity of liver cathepsin in fasted or protein-depleted rats was unchanged. In contrast, Yamamoto ('53) reported that this enzyme decreased in rats fed a low protein diet.

The activity of liver alkaline phosphatase in rats receiving the diet containing different levels of protein for 15 days did not change appreciably throughout all these levels. It was shown, however, that the activity of this enzyme in rats fed a protein-free diet for 29 days was maximal (an increase of 1.4-fold from the minimal value), and decreased to the minimum in rats fed the 25% casein diet (fig. 6). A similar observation was reported by Ross and Batt ('56), who noted that the minimal activity of liver alkaline phosphatase was obtained in rats fed a 30 to 40% casein diet, but the degree of the change obtained in our experiments was markedly less than that observed by Ross et al.

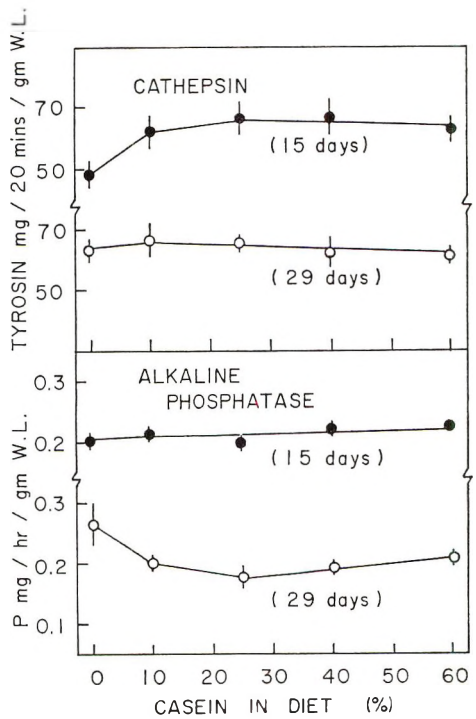

Fig. 6 Effect of different levels of protein on the activities of liver cathepsin and alkaline phosphatase in rats (15 and 29 days' feeding).

The reason is uncertain for the response curves of the activities of cathepsin and alkaline phosphatase of rats fed for 15 or 29 days differing from each other.

DISCUSSION

From the results reported here it is possible to divide the liver enzymes examined into about 4 types with respect to the response curves plotted against the dietary level of casein.

The enzymes in the first type are xanthine oxidase, succinic dehydrogenase and D-amino acid oxidase, the activities of which, after an initial rise, reached the maximal value and then showed a plateau or a slight increase with increasing dietary protein.

Arginase, uricase, glutamic - pyruvic transaminase, and glutamic - oxaloacetic transaminase are the enzymes of the second type, the activities of which increased in the order of the increasing protein content of the diet, although the response curves of arginase and other enzymes to the level of dietary protein were not exactly the same.

The enzyme of the third type is cathepsin, which failed to change much at all protein levels.

The enzyme of the fourth type is alkaline phosphatase (29 days feeding), which showed the maximal value with a protein-free diet, decreased with increasing the protein level from zero to 25% and led to the minimal value with about 25% of casein in the diet. Ribonuclease, for which the maximal activity was observed in rats fed a protein-free diet (Zigman and Allison, '59), may be considered as one of the enzymes belonging to this type.

Among the liver enzymes examined, the response curves of enzymes of the first type plotted against the protein levels in the diet are similar to that of liver nitrogen content plotted in a similar way. But the decreases in enzyme activities of the first type in rats fed protein-free or low protein diets, in general, are much more striking than that of liver nitrogen.

It would be expected that many liver enzymes that decrease with a protein-free or low protein diet belong to the first or second type. It seems also that most liver enzymes may be included in these 4 types.

Comparison of the response curves of liver enzyme activities and of the growth rate to the protein level in the diet, leads to consideration of the possibility that there may be a correlation between the response curves of liver xanthine oxidase, succinic dehydrogenase and glutamic dehydrogenase (belonging to the first type) and the growth response in rats, because the maximal growth was obtained in rats receiving the 25% casein diet, at which level the activities of these liver enzymes each reached maximal value. Recently, Zigman and Allison ('59) and Allison et al. ('61) reported that the rates of growth of rats fed different dietary proteins of varying nitrogen intakes were correlated with serum, kidney and liver ribonuclease activity. It is presumable that such studies may give some insight into the relationship of growth and protein metabolism in the body.

The physiological meaning of the changes in these enzymes with dietary protein level is not clear, but that of the changes in some enzymes is under investigation in our laboratory.

SUMMARY

1. The activities of 10 liver enzymes were studied in rats fed diets containing various levels of casein for 15 or 29 days. The enzymes studied were xanthine oxidase, uricase, arginase, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, cathepsin, D-amino acid oxidase, glutamic dehydrogenase, succinic dehydrogenase and alkaline phosphatase.

2. It was found that these liver enzymes could be divided roughly into 4 types with respect to the response curves of these activities obtained by plotting against the dietary protein level.

3. The enzymes of the first type decreased in rats fed protein-free or low protein diets, but did not change greatly after reaching the maximal value with increasing dietary protein levels. These enzymes were xanthine oxidase, succinic dehydrogenase, glutamic dehydrogenase and D-amino acid oxidase. The enzymes of the second type increased with each increase in the dietary protein level; these were arginase, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase

and uricase. The enzyme of the third type, cathepsin, was essentially unchanged in rats fed diets containing different dietary protein levels. The enzyme of the fourth type, alkaline phosphatase, increased in rats fed protein-free or low protein diets, (29 days feeding).

4. It was shown that the response curves of enzymes included in the first type as liver xanthine oxidase, succinic dehydrogenase and glutamic dehydrogenase were similar to those of liver nitrogen and of the growth rates in animals.

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Gamma Globulin Absorption in the Baby Pig: THE NONSELECTIVE ABSORPTION OF HETEROLOGOUS GLOBULINS AND FACTORS INFLUENCING ABSORPTION TIME[1]

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At the time of birth the baby pig is virtually devoid of serum gamma globulin and the only natural source of immune bodies is colostrum. A number of techniques have been used to determine the duration of the period of gamma globulin absorption through the intestinal mucosa. Young and Underdahl ('49) determined the hemagglutinin inhibitor titer in baby pigs nursing sows immunized to swine influenza and concluded "that after about 8 hours, some physiological change occurred in the intestinal epithelium that retarded further absorption of hemagglutinin inhibitor substances, or if they were absorbed they became fixed to tissues so that the circulating titer did not show a further increase." Other studies have shown that little absorption occurred after 24 hours. Brambell ('58) reported that the postnatal time of transmission of passive immunity in the pig was 36 hours. Nelson ('32), Barrick et al. ('54), Hoerlein ('52), Speer et al. ('59), Olsson ('59) and Lecce and Matrone ('60) concluded that no measurable intestinal absorption occurs in the baby pig after it is 48 hours old. Although much of the work concerned with absorption of antibodies by baby pigs has been quantitative in nature, Olsson ('59) and Lecce et al. ('61) have studied the nonselective absorption of heterologous antibodies.

The purpose of this study was to examine the nature of gamma globulin absorption and particularly to determine what factors are involved in the cessation of absorption.

PROCEDURE

Seventy-one of the pigs used in this study were farrowed naturally and are designated as "FN" pigs. Thirty-six were taken from sows by hysterectomy and reared in isolation. These animals, deprived of colostrum and virtually devoid of serum gamma globulin are designated as "HI" pigs. Colostrum was obtained from sows at the time of parturition by hand milking after the intravenous injection of 1 ml (10 units) of oxytocin. Collections averaged 300 to 400 ml from each sow.

Blood samples were collected via anterior vena cava or cardiac puncture, allowed to clot, centrifuged and serum samples obtained. Paper electrophoretic patterns were prepared as follows: 0.01 ml of serum was applied to a strip of Schleicher and Schull 2043 A paper placed in a Spinco-Durrum[2] type cell (17 hours, 5.0 ma current) using a barbital buffer of 0.075 ionic strength and pH 8.6. Patterns were stained with 0.1% bromphenol blue or 0.1% light green dye (FD and C no. 2) in methanol. Strips were scanned and integrated with a Spinco-Analytrol.[3] This instrument with a 600 mμ filter is approximately three times as sensitive to the light green dye as it is to bromphenol blue when a 500-mμ filter is used.

Electrophoretic separations were made in polyacrylamide gel by the method of Raymond and Wang ('60) using a buffer solution composed of trishydroxymethylaminomethane, 60.6 gm; disodium ethylenediamine tetraacetic acid, 7.8 gm; and boric acid, 4.6 gm, in a volume of 2 liters (pH 8.9 to 9.1). One hundred milliliters of the buffer were diluted to 300 ml with

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[2] Beckman Instruments, Inc., Fullerton, California.
[3] See footnote 2.

water and a 5% acrylamide gel prepared in the diluted solution. Immuno-diffusion on cellulose acetate was by the method of Consden and Kohn ('59).

Colostrum was tagged with fluorescein isothiocyanate (FITC) as follows: pooled colostrum samples were centrifuged for 30 minutes at 4°C at 4,000 × *g*. The fat layer was mechanically removed and the whey diluted 1:1 with 0.5 M carbonate-bicarbonate buffer solution (pH 9.0). Protein concentration was determined by the method of Lowry et al. ('51) and amorphous FITC (50 mg per gm of protein) was added and stirred in for 18 hours at 4°C and centrifuged. The supernate was dialyzed in a buffered solution of pH 7.0 until all residual FITC was removed (4 to 5 days).

Fluorescent gamma globulin was fractionated as follows: the undialyzed fluorescent colostrum was diluted fourfold with distilled water and the pH adjusted to 4.5 with 0.4 M HCl. The resulting precipitate of casein was removed by centrifugation and the pH of the solution adjusted to 6.0 with 0.5 M NaOH. Ammonium sulfate was added to the solution to 0.4 saturation (1.64 M). After standing overnight in a refrigerator the precipitated globulin was removed by centrifugation and washed three times with 1.64 M ammonium sulfate solutions. The precipitate was redissolved in buffered saline and dialyzed against the same saline to remove any remaining ammonium sulfate and unconjugated FITC.

Isolated HI pigs were pan fed water or cow's milk fortified with minerals and vitamins according to the formula developed by Underdahl and Young ('57). Test solutions were administered with a 50-ml syringe and stomach tube or injected directly into the exposed small intestine of pigs anesthetized with pentobarbital sodium (30 mg per kg). Intestinal tissues were prepared for microscopic examination by frozen-tissue sectioning techniques or by the routine formalin-paraffin method. A Leitz Ortholux UV microscope with either a UV (2mm UG-1) lamp filter and a 215-mm Euphos barrier filter or a BG 12 lamp filter with a OG 1 barrier filter was used for fluorescent microscopy. Photomicrographs were made with Hi-speed Ektachrome film at exposures of two to 6 minutes or Panatomic-X film.

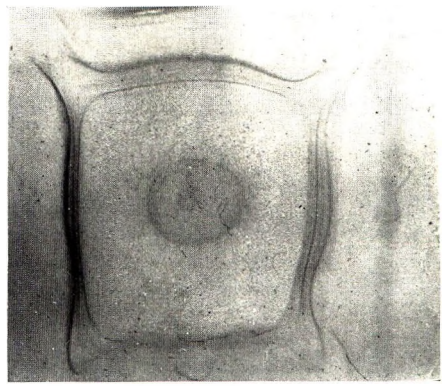

Fig. 1 Immuno-diffusion pattern for the detection of gamma globulin in baby pig serum. Center spot, antigen of pig origin: peripheral spots, antibody of rabbit origin.

Experiment 1. Gamma globulin levels in the serum of newborn pigs. The presence of small but consistent amounts of gamma globulin in the sera of pigs prior to nursing have been demonstrated in this laboratory. In these studies, quantitative determinations showed the average amount of gamma globulin in the serum of 87 pigs prior to feeding was 2.1 mg per ml with a range of 1.3 to 3.8 mg per ml. Qualitative determinations were made using the technique of immuno-diffusion on cellulose acetate. Six rabbits were immunized at weekly intervals against gamma globulin fractionated from swine colostrum or serum. Immuno-diffusion patterns were positive for gamma globulin in the serum of newborn pigs when diffused against rabbit serum of both sources (fig. 1).

Rutqvist ('58), Nordbring and Olsson ('57), Brambell ('58) and Lecce and Matrone ('60) have shown the baby pig to be devoid of serum gamma globulin at the time of birth. Olsson ('59) using column electrophoresis, observed small amounts (1.3 mg per ml) of gamma globulin present in the cord serum of baby pigs.

These differences may be explained by comparing the methods used to detect the presence or absence of this serum fraction. The greater sensitivity of the Spinco-Analytrol to light green dye than to either bromophenol blue or amido black allows the quantitative determination of low concentrations of gamma globulin (fig. 2)

and the use of polyacrylamide gel in the electrophoretic separation of serum confirms this finding.

Experiment 2. Comparison of serum patterns of nursing pigs with pigs fed an artificial diet. Five FN pigs were allowed to nurse the sow for 30 days after birth. Five HI pigs were raised in isolation and fed cow's milk for the same period. Blood samples were taken from the anterior vena cava on days 1, 2, 3, 4, 10, 18, and 30. Total serum protein, albumin, alpha, beta and gamma globulin concentrations were obtained from protein determinations and electrophoretic patterns.

The greatest differences between the serum profiles of the nursing piglets compared with those from piglets fed a modified cow's milk (fig. 3) are in the total serum protein level and the amount of gamma globulin present. The 6% protein level of the nursing pig is composed of 33 mg per ml of gamma globulin, whereas the 2.4% total protein level in the HI pig contains 2.8 mg per ml of gamma globulin. The other change noted during the first 30 days is the relatively large increase in the serum albumin of the HI pigs. These values are in agreement with those reported by Lecce and Matrone ('61) with the exception of the initial amount of gamma globulin present in the newborn.

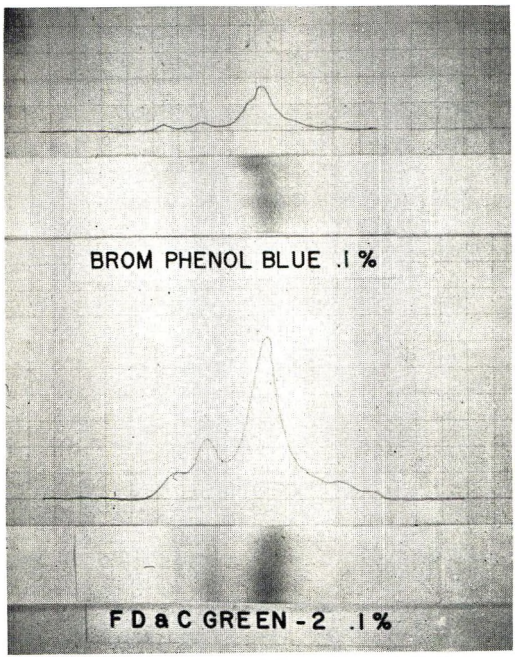

Fig. 2 Comparison of the sensitivity of bromphenol blue and light green dyes on paper electrophoretic patterns.

Experiment 3. Nonselective absorption of heterologous gamma globulins. In an attempt to find a suitable substitute for swine colostrum, 12 HI pigs were tube-fed bovine, ovine and human colostrum. Because of species differences in the concentration of gamma globulin (bovine, 12.2 mg per ml; ovine, 51.4 mg per ml; human, 4.68 mg per ml; and porcine, 57.6 mg per ml), the concentration of gamma globulin from these sources was adjusted by dilution or evaporation to 30 mg per ml. Each pig received 100 ml of one of these solutions via stomach tube. This approximates 60 ml of natural swine colostrum (3.45 gm per kg of body weight). Serum samples were obtained 24 hours after feeding and total protein concentrations and paper electrophoretic patterns determined.

The results of this experiment indicated that only the gamma globulin of swine origin was absorbed by the newborn pig. When this experiment was repeated, however, using the more delicate methods of polyacrylamide gel electrophoresis, immuno-diffusion or fluorescent microscopy, absorption of gamma globulin from the above sources was evident.

Commercial preparations[4] of globulins from equine, bovine, human and porcine serums were dissolved in buffered saline solutions, (20 mg per ml) tagged with FITC and injected directly into 5.0-cm intestinal segments of 6-hour-old pigs for subsequent fluorescent microscopy. Beta (fraction III) globulins from bovine and porcine serum and the alpha globulin (fraction IV) from bovine serum were treated in the same manner.

Fluorescent microscopy has proved that the gut of the baby pig is nonselective in its absorption, in that all of the above preparations were taken up by the intestinal mucosa (fig. 4). A comment should be made concerning the actual absorption of FITC tagged gamma globulin compared with the uptake of FITC alone. When solutions of fluorescein isothiocyanate were injected directly into the small intestine and subsequently examined by fluorescent

[4] Pentex Incorporated, Kankakee, Illinois.

microscopy, there was only a slight staining of the striated border of the epithelial cells, and no evidence of intracellular fluorescence.

Experiment 4. Factors influencing absorption time. Twelve FN pigs (group 1) were allowed to nurse the sow during the first 106 hours after birth. Fifty-four pigs were isolated from the sow before nursing and given the following treatments: group 2 was pan-fed 1.5 ounces of cow's milk twice daily; group 3 was pan-fed water twice daily, and group 4 was starved from the time of birth to the termination of the experiments. Two or more pigs from each group were given FITC-tagged gamma globulin of colostrum origin directly into the small intestine at 12, 36, 60, 84 and 106 hours after birth. Intestinal segments were removed from each pig at 1, 3, 5, 7, and 9 hours after administration of the globulin. Fluorescent microscopic examinations were made to determine the degree of cellular absorption.

The results of this experiment are perhaps the most informative of those concerned with factors determining the postnatal absorption time. In table 1 the absorption level in the 4 groups of pigs being fed or starved is shown qualitatively. Those pigs receiving sow's colostrum or cow's milk did not absorb gamma globulin after 12 hours of feeding, whereas pigs receiving only water or pigs that were starved for periods up to 106 hours after birth still retained the ability to absorb gamma globulin. A photomicrograph of intestinal tissue examined under ultraviolet light before administration of any fluorescent compound is shown in figure 5A. Tissue typical of those pigs receiving colostrum or cow's milk prior to globulin administration is illustrated in figure 5B. The fluorescent gamma globulin is re-

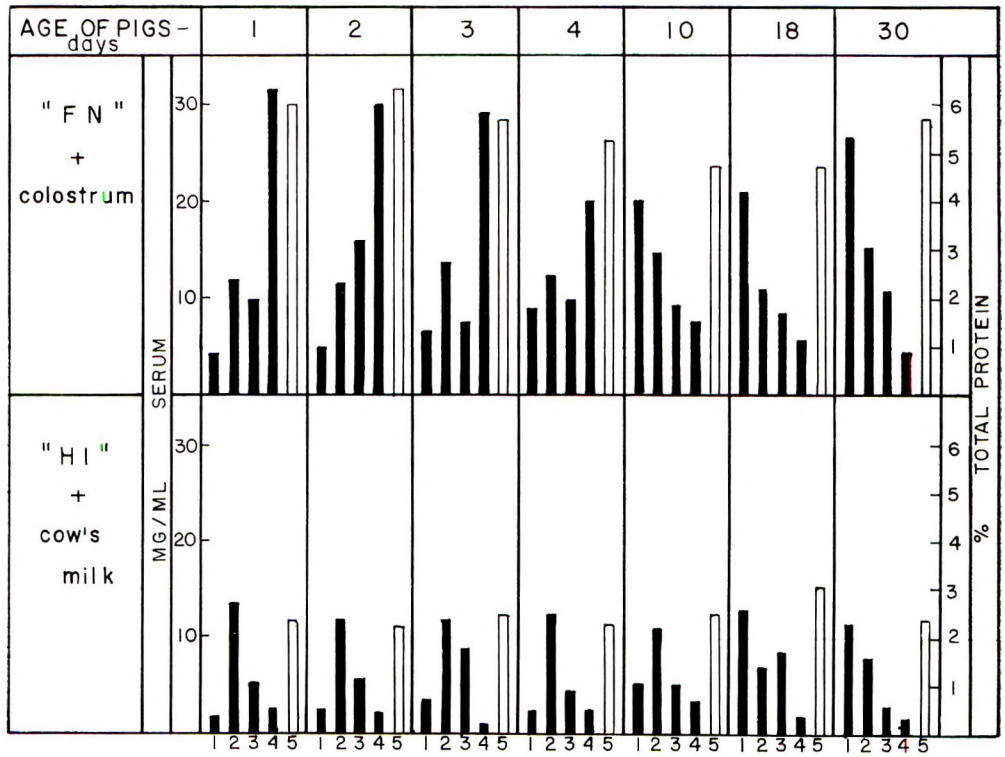

Fig. 3 A comparison of the serum protein profiles of pigs farrowed and nursed (FN) with pigs taken by hysterectomy, isolated (HI) and fed cow's milk.

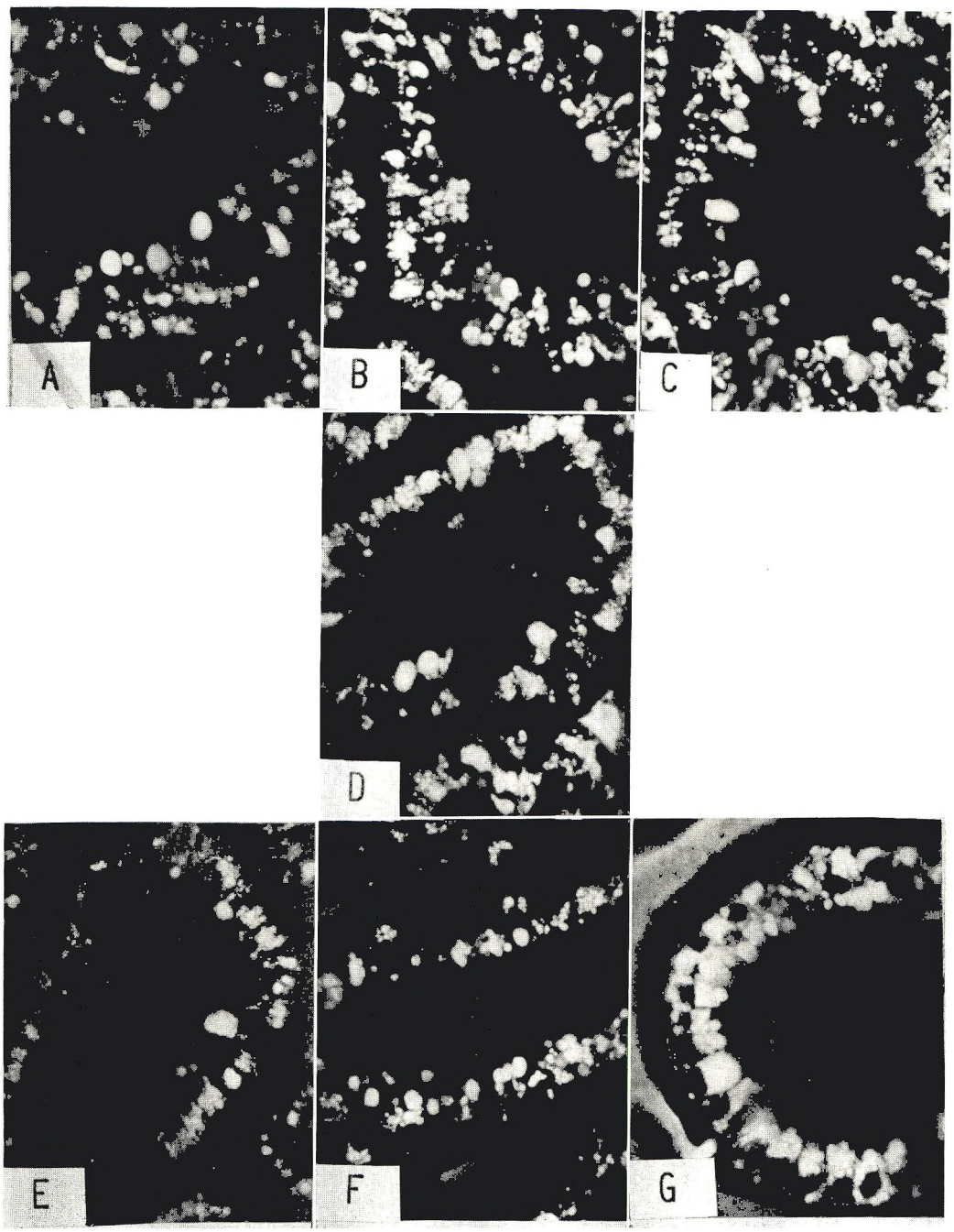

Fig. 4 Nonselective absorption of heterologous FITC-tagged globulins by intestinal epithelial cells in the baby pig. (× 500.) Panatomic film-90 sec. exposure.

A. Porcine colostrum	γ-globulin	E. Bovine serum	γ-globulin
B. Porcine serum	γ-globulin	F. Equine serum	γ-globulin
C. Porcine serum	β-globulin	G. Human serum	γ-globulin
D. Bovine serum	α-globulin		

TABLE 1

Effects of feeding and starvation on the absorption of gamma globulin in the baby pig

Group no.	Age of pigs (hours)[1]				
	12	36	60	84	106
1 Sow's milk	–[2]	–	–	–	–
2 Cow's milk	–	–	–	–	–
3 Water	+[3]	+	+	+	+
4 Starved	+	+	+	+	+

[1] Intestinal segments taken 7 hours after the administration of gamma globulin from sow colostrum.
[2] – = no absorption.
[3] + = active absorption.

tained between the villi and there has been no cellular absorption. The same results are seen in sections taken from pigs 12 hours through 106 hours after birth. Tissue taken from a pig starved for 106 hours before administration of the FITC-tagged globulin is shown in figure 5C. The marked fluorescence within the epithelial cells of the villi is the result of gamma globulin absorption.

DISCUSSION

The presence of small but consistent amounts of gamma globulin was demonstrated in the sera of newborn pigs and those taken by hysterectomy. Electrophoretic separation of serum in polyacrylamide gel confirmed this observation. Gamma globulin was also identified in serum of newborn pigs by immuno-diffusion on cellulose acetate paper (fig. 2). The antibody used in the latter technique was serum from rabbits immunized against swine serum or swine colostrum gamma globulin. These results are in agreement with those of Olsson ('59) who reported small amounts (1.3 mg per ml) of gamma globulin in cord serum. In our studies, the average amount of gamma globulin observed in serum from 87 pigs prior to feeding was 2.1 mg per ml with a range of 1.3 to 3.8 mg per ml. Rutqvist ('58) and Lecce and Matrone ('60), using conventional paper electrophoretic methods, did not find gamma globulin in either fetal or neonatal pig serum. The greater sensitivity of the Spinco-Analytrol to light green dye than to bromphenol blue or amido black allows the quantitative determination of the low concentrations of gamma globulin in the serum of newborn pigs.

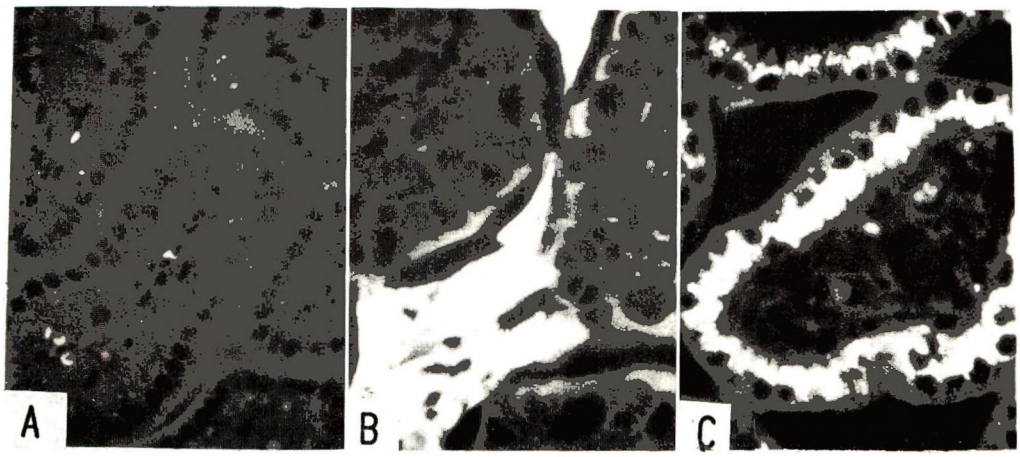

Fig. 5 Effects of feeding and starvation on absorption of fluorescent gamma globulin in the baby pig.

A. Photomicrograph of intestinal tissue before administration of gamma globulin.

B. Photomicrograph from a pig which had nursed 24 hours before administration of gamma globulin.

C. Photomicrograph from a pig starved for 106 hours before administration of gamma globulin. (Tissues removed 6 hours after administration of FITC-tagged gamma globulin. Figures taken from 500 × Hi-speed Ektachrome slides, 3 min. exposure time.)

The results of the second experiment are self-evident and in agreement with the values reported by Lecce and Matrone ('61). The predominating selectivity of the small intestine of the baby pig for the absorption of gamma globulin and the apparent rejection of other protein fractions of colostrum is seen in figure 1. These pigs taken by hysterectomy and raised in isolation are considered to be "disease-free" and are not "germ-free." Hence, any increase in the gamma globulin of those pigs may be due to the normal biological activity of the animal or to an exposure to incidental bacteria or "foreign" protein in the diet.

Initial experiments in this laboratory on the absorption of heterologous antibodies, using paper electrophoresis and bromphenol dye, indicated that only the gamma globulin from swine was absorbed, whereas gamma globulin from human, bovine and ovine colostrum was not. Later studies using fluorescent-tagged fractions from the above sources, as well as gamma globulin from the serum of the canine, bovine, human, equine and swine showed absorption of all of these globulins, irrespective of source. These results confirmed the work of Olsson ('59) showing the absorption of equine serum gamma globulin and the work of Barrick et al. ('54) showing the absorption of gamma globulin from swine and bovine serum. Lecce et al. ('61) studied the absorption of unaltered nonporcine proteins using cow's colostrum, cow's milk fortified with avian eggs and cow's milk fortified with polyvinylpyrrolidone.

Some of the confusion concerned with the absorption of gamma globulin in the pig may be caused by the numerous methods used to determine serum levels and the failure to report the exact amount of gamma globulin administered or determined in serum. Olsson ('59) reported that the inability to demonstrate absorption of gamma globulin depends upon factors other than the pig's ability to absorb immune globulins from either homologous or heterologous serum, one of which is the total amount of protein or gamma globulin administered. Unpublished data from this laboratory show that absorption of gamma globulin is directly proportional to the amount given, reaching the maximal level when 60 ml of whole swine colostrum is administered. This gradient may hold true for the nursing pig also; that is, one may assume the pig has nursed for a period of hours, when in reality it has nursed very little. Likewise a large pig may obtain a small amount of colostrum at the same time a small pig may ingest a large amount per unit of body weight. Standardization of the amount of gamma globulin given, the method of administration and the technique of detection and reporting may help to clarify these misinterpretations.

The most striking results of these experiments concerned the effects of feeding as compared with starvation. The pigs that were allowed to nurse immediately after birth, or that were fed modified cow's milk, exhibited complete cessation of absorption 12 hours postnatally. Marked absorption was evidenced in those pigs receiving only water or in those starved for 106 hours postnatally. Just how long a pig will retain the ability to absorb gamma globulin during a period of starvation remains to be determined. In future studies either feeding or intraperitoneal injections of dextrose solutions, may prolong this period of starvation.

The discrepancies between the reports of other workers and the results of this experiment were directly concerned with the feeding of control animals. In all reports reviewed, dealing with the period of absorption and the cessation of the same, no studies were found in which the control animals had been starved. Pigs were fed either sow colostrum, cow colostrum or cow's milk. Hence, previous reports of the length of time that absorption will take place in the newborn pig as being 24, 36, or 48 hours are correct for pigs that have been fed milk, but are incorrect for the starved pig.

From the studies of milk from either the sow or the cow, it is apparent that absorption of gamma globulin is an "all-or-none" phenomenon. That is, once the epithelial cell of intestinal mucosa has filled with gamma globulin (or perhaps any soluble protein) further absorption ceases. Although photomicrographs of intestinal tissues taken 6 hours after the administration of gamma globulin to pigs 106 hours after birth show absorption in the starved ani-

mal and rejection in the milk-fed animal, the same results are seen at any period of tissue sampling. Plans for the future incorporate the study of purified fractions of serum and colostrum proteins administered to baby pigs and the resulting effect upon gamma globulin absorption.

SUMMARY

Serum profiles for total serum proteins, albumin, alpha, beta and gamma globulin in baby pigs taken at 5 intervals up to 31 days of age were given. These values for the naturally farrowed-nursing pig were compared with values from pigs obtained by hysterectomy and raised in isolation with a modified cow's milk.

Using fluorescent-tagged gamma globulins from various sources, (bovine, human, equine and porcine) as well as the alpha fraction from bovine serum and the beta fraction from bovine and swine serum, the absorption of heterologous gamma globulins was confirmed.

Pigs that were fed milk for 12 hours after birth failed to absorb tagged gamma globulin after that length of time. Pigs that received only water or that were starved for 106 hours subsequent to birth still retained the ability to take up gamma globulin.

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Studies on Iodine Nutrition in Thailand

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For some years the medical profession of Thailand has been aware that goiter was highly prevalent in the northern provinces and to a lesser degree in the northeastern areas of the country. During the years 1937–1951, Sem Pring-puang-geo ('55) made extensive clinical studies, particularly in Chieng Rai Province, and delineated a "goiter belt," extending from Burma through the northern provinces of Thailand, over to the northeastern areas, and on into Laos. In 1956 Dr. V. Ramalingaswami was invited by the World Health Organization (WHO) to survey certain areas in Thailand with a view to intensifying activity in the fields of beriberi and goiter control. Ramalingaswami ('60) surveyed school children and adults in the provinces of Chieng Mai, Chieng Rai, Ubol and Udorn, where the incidence of goiter was found to be 58, 58, 21 and 15%, respectively. This initial survey prompted WHO to establish a program under the direction of Dr. J. V. Klerks ('60), who extended the scope of the goiter studies to three other northern provinces: Prae, Uttaradit and Lampang. In each of these areas the prevalence of goiter in school girls was above 40%.

When, in 1959, the Kingdom of Thailand requested that the Interdepartmental Committee on Nutrition for National Defense conduct a joint nutrition survey, it was apparent that goiter was one of the important problems that would be encountered. Hence, it was decided to supplement the data on goiter, which would be gathered by the usual clinical examinations, with special studies on iodine metabolism, for it was realized that studies of goiter prevalence, except in younger age groups, do not provide data concerning the current state of iodine nutrition — particularly in those individuals who have no, or only border-line, thyroid enlargement.

Preliminary contacts were therefore made with Dr. Sem Pring-puang-geo, Director of the Women's and Children's Hospital, Bangkok, and with Dr. Romsai Suwanik, Department of Radiology, Siriraj Hospital, University of Medical Sciences, Bangkok. Drs. Sem and Romsai have made valuable contributions to the study of goiter and iodine metabolism in Thailand. Only through the cooperation of Drs. Sem, Romsai, and their associates could the studies reported herein have been completed.

METHODS

The purpose of this study was to evaluate the current status of iodine nutrition in selected segments of military and civilian populations. It has been recognized since the initial goiter studies of Marine and Kimball ('17) that the most sensitive group in any population sample is the adolescent female. Hence, it was decided to restrict the civilian study to this group. Moreover, an attempt was made to evaluate only those individuals who had no goiter or at most grade 1, i.e., slight, enlargement. In each area visited, after approval by the Governor of the Province, contacts were made with the principals of selected schools in urban and rural areas. A visit was made to schools in each area in the morning and afternoon; the thyroid glands of the students in all rooms were observed and palpated by a single examiner. A method of recording similar to that described by Muñoz and Anderson ('59) was used. The WHO

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system of classification was followed (Perez et al., '60). At approximately 10:00 AM or 2:00 PM, a group of girls, on an average 15, was picked at random. They were asked to drink a dose of approximately 5 μc of radioactive iodine[1] (I^{131}), dispensed by an automatic pipette into a plastic cup and diluted with water. The cup was rinsed with water and the rinsings were swallowed. The next morning or afternoon a second visit was made to the school. Apparatus was set up so as to be ready to begin counting at approximately 9:00 AM or 2:00 PM. Hence, the values for the uptake represent 24 hours plus or minus one hour. At this second visit casual urine specimens were obtained in paper cups; these were transferred to amber bottles containing toluol. Samples of military groups were processed in similar fashion.

A Nuclear Chicago portable decade scaler (no. 2800) was used in all studies. This instrument contains a storage battery that can be recharged whenever necessary. It weighs 30 pounds and hence is readily portable. A transistorized scintillation detector (Nuclear Chicago, Model DS5-2) with a one-inch sodium iodide crystal and a flat field collimated nose piece was used. This was mounted on a wooden block attached to the base of a laboratory jack (Cenco-Lerner, 19,089). A plastic tube, which was made to fit over the end of the collimator, was of such a length that its end was exactly 25 cm from the face of the sodium iodide crystal. This end was placed against the neck so as to cover the thyroid region. The subject was seated in a straight-back chair to which was clamped a dental type head-rest. The head was positioned so that the neck surface was parallel to the plane of the plastic tube's opening (fig. 1).

Each week appropriate dilutions of I^{131} were made so that the dose to be administered was contained in 1 cm^3 of water. The amount was gauged so that the rate of decay would furnish a dose of 5 μc at mid-week. One cubic centimeter of the dose was diluted to 20 cm^3 in a volumetric flask. This standard was counted at 25 cm from the surface of the crystal, i.e., on a plane with the end of the plastic tube. This standard had been calibrated with a plastic neck phantom (Brucer, '59) so as to obtain correction factor for scatter and absorption. This factor, 0.84, was multiplied by the count obtained over the neck.

[1] Radioactive iodine was obtained from the Radiochemical Center, Amersham, England, through the cooperation of Dr. Romsai.

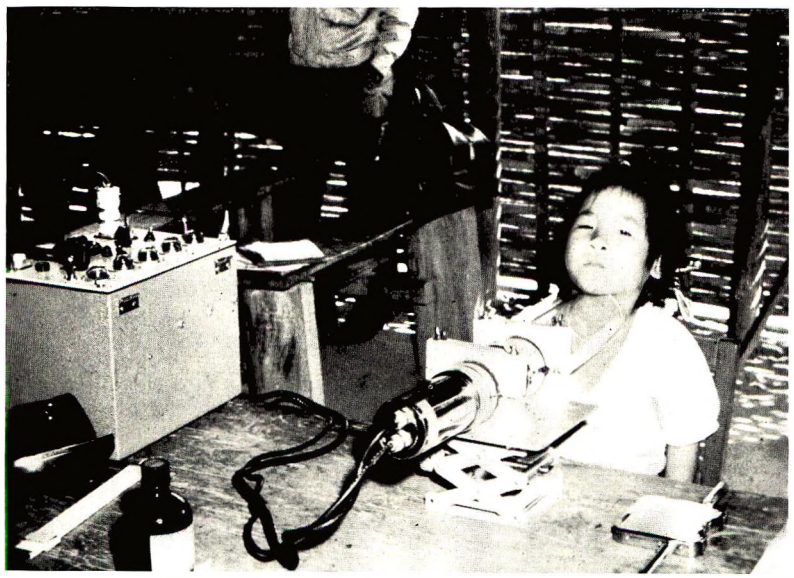

Fig. 1 Scene in village school house at Huay Kae, Prae Province, showing technique for radioiodine uptake measurement.

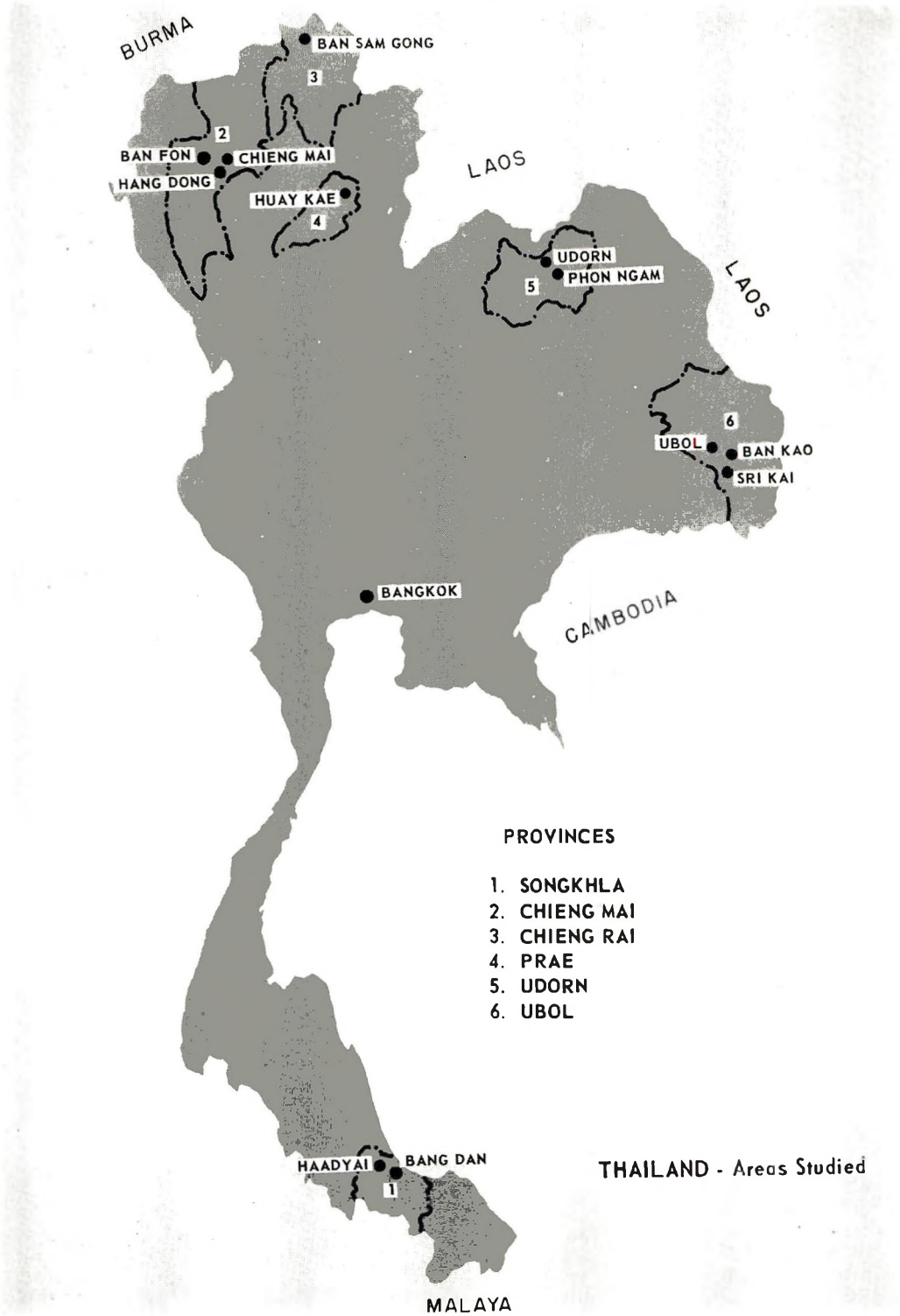

Fig. 2 Map of Thailand showing provinces visited and town and village sites where samples of school girl and military populations were studied.

Two counting intervals of two minutes each were made with the neck unshielded; two more were made with a 0.5-inch lead shield between the neck and plastic tube for background levels, according to the procedure outlined by Brucer ('59).

The creatinine content of the urine samples was determined by the alkaline picrate method. Iodine determinations[2] were performed by using a modified Zak method.[3]

In all, studies of I^{131} uptake were carried out on 135 school girls at 10 different sites (fig. 2) and on 69 army personnel at 5 sites. Determinations of iodine in the urine were performed on 117 school girls and 46 army personnel. The decrease in numbers is due to insufficient volumes, breakage, or loss from other causes.

RESULTS

Data on the 24-hour radioactive iodine uptake by the thyroid glands of samples of school girls in 10 sites in the 6 provinces which were visited are presented as histograms in figures 3–7. The means and standard deviations are presented in table 1. Mean values below 50% were observed at three sites: both groups in Songkhla Province and at the Udorn town school. All the rest had mean uptakes above 50% which, of course, indicates an increased affinity of the thyroid gland for administered iodide. The normal mean for radioactive iodine uptake should fall within the range of 30 to 45%.

In table 1 are shown mean values for urinary concentration of stable iodine expressed as micrograms (μg) per gram of creatinine. It will be noted that in only three areas, those mentioned previously, are values found to be above 50 μg of iodine per gm of creatinine. Included here are values for 19 school girls from a parochial school in Washington, D. C.; the mean value is 123.5. None had values below 50

[2] Performed by the Boston Medical Laboratory, Inc., Boston.

[3] Benotti, J., and N. Benotti 1961 Personal communication.

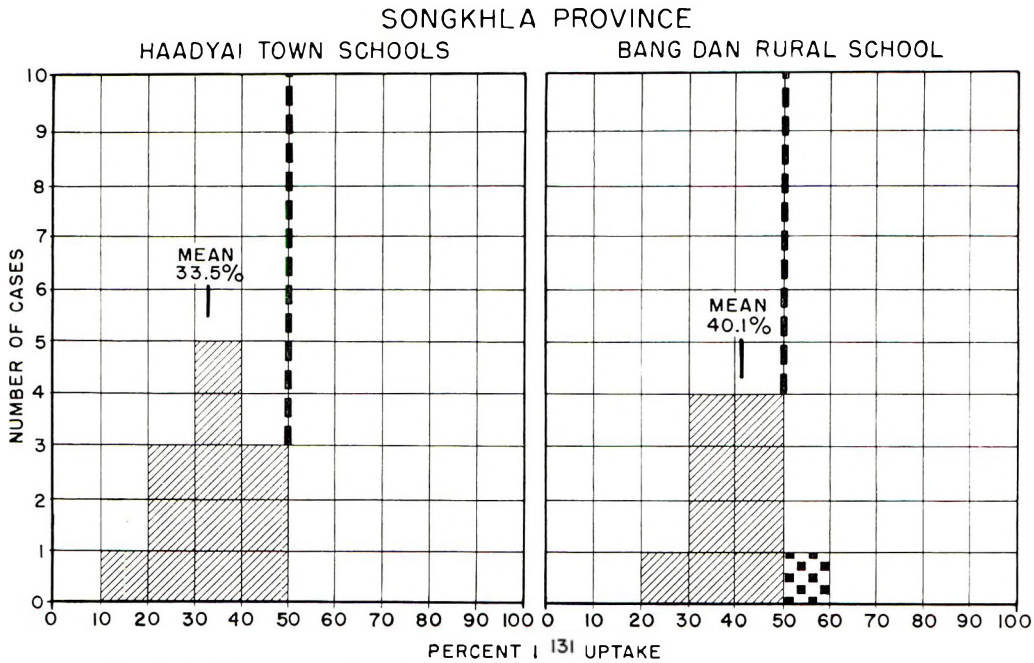

Fig. 3 Frequency histograms of radioiodine uptake in two areas in Songkhla Province, located in the southern portion of the peninsula of Thailand, at latitude 8°N. The land is flat and bordered on the northeast by the Gulf of Thailand. The remainder borders on Thailand or Malaya. Haadyai town has a population of approximately 10,000, many of whom are Chinese. The school at Bang Dan is located just off the main road between Haadyai and Songkhla towns. The population has available an abundance of fresh marine animal foodstuffs.

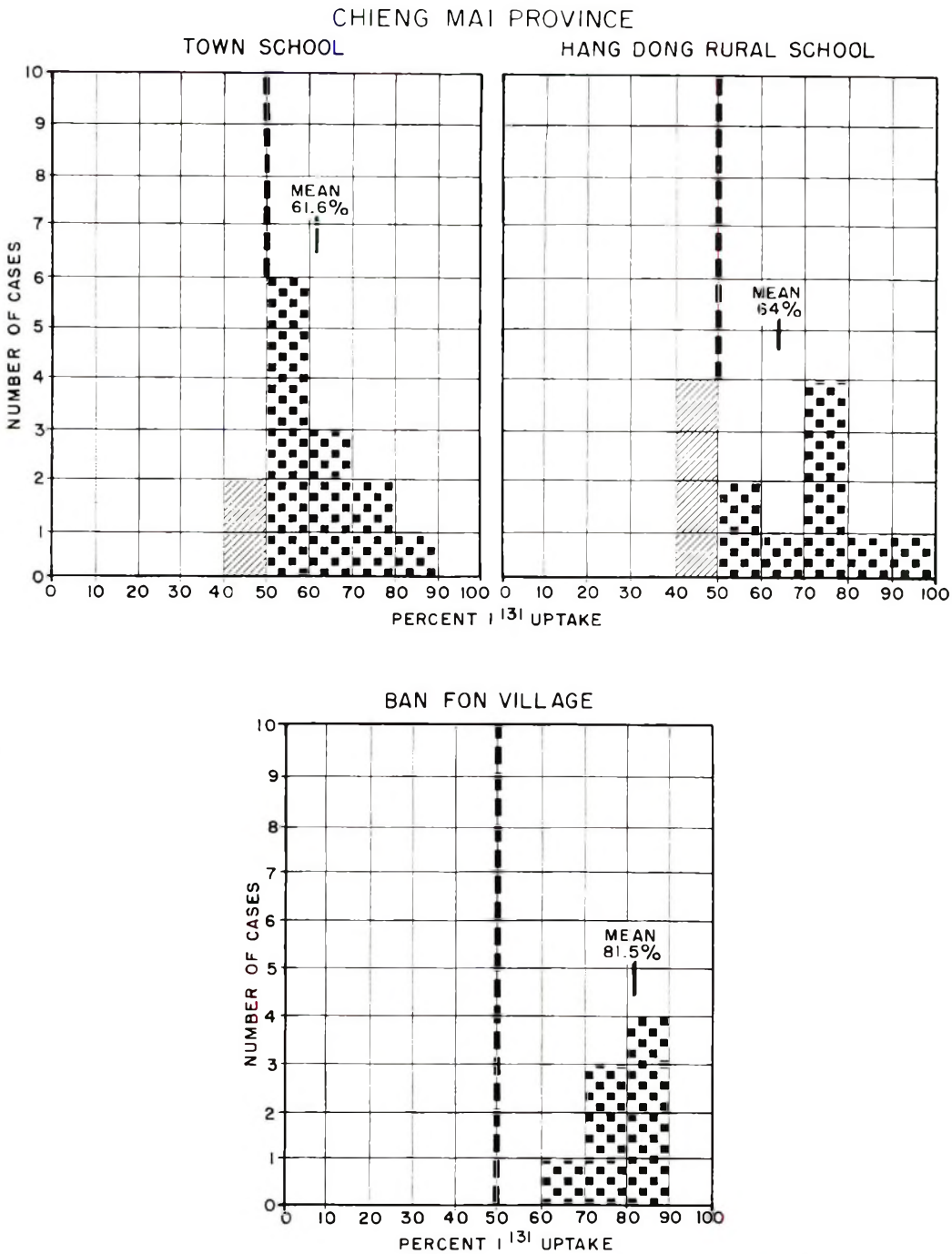

Fig. 4 Frequency histograms of radioiodine uptake in three areas in Chieng Mai Province, located in the northwest portion of the country, at latitude 18°N. The land is traversed from north to south by mountain chains which represent extensions of higher formations in Burma, Laos, and southwestern China. Between the mountains are valleys where rice fields are found. Chieng Mai town is the second in size in Thailand, population about 60,000. Hang Dong school is off the main road south from Chieng Mai town and draws children from villages near this road and farther away. Ban Fon village is about 10 km by poor road from the main highway.

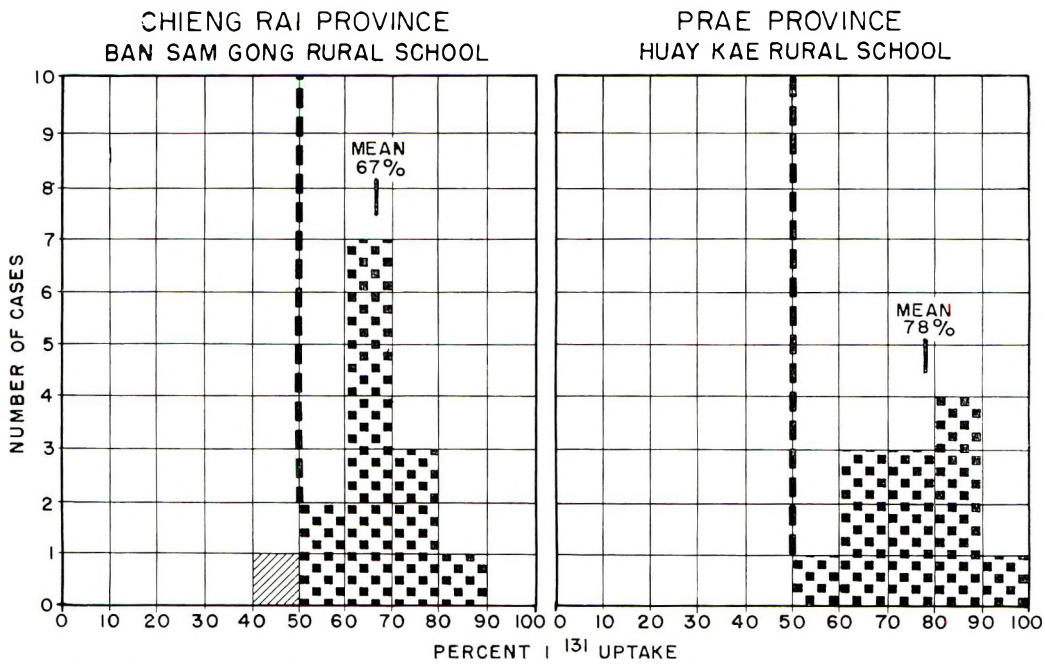

Fig. 5 (a) Frequency histogram of radioiodine uptake from a village school in Chieng Rai Province is in the northernmost portion of the country at latitude 20°N abutting on Burma and Laos. It is more mountainous than Chieng Mai. Ban Sam Gong school is on the main road north from Chieng Rai town to the Burma border. The children come from surrounding villages. (b) Frequency histogram of radioiodine uptake from a village school in Prae Province which is south of Chieng Rai at latitude 18°N. The country is hilly. Huay Kae school, a two-room building (see fig. 1), is on the main road from the towns of Prae to Nan in the northeast.

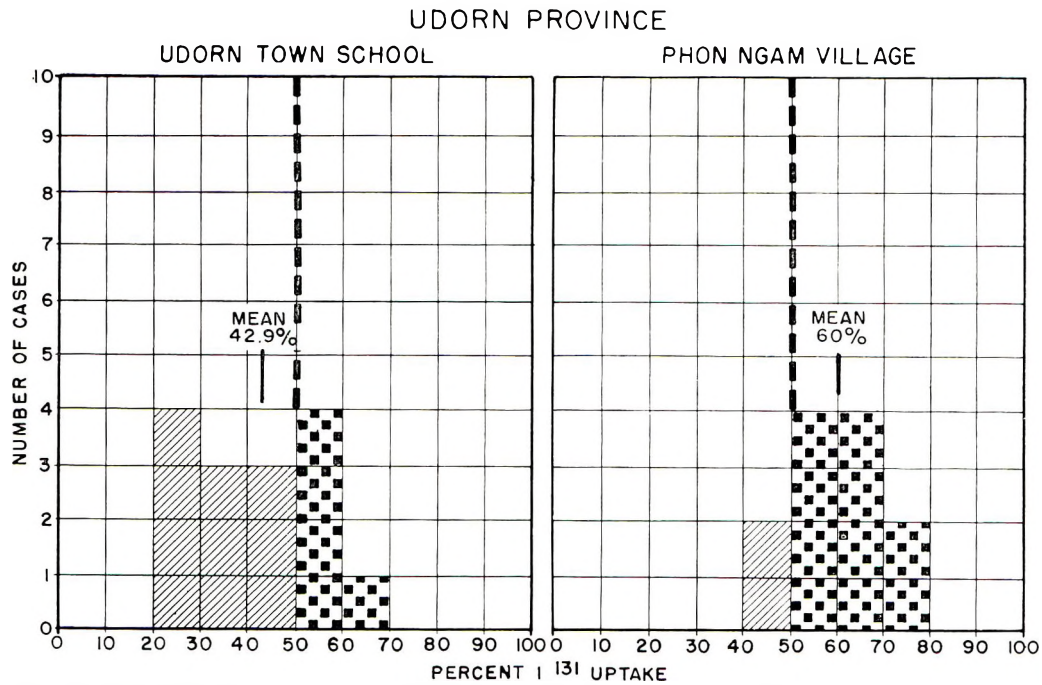

Fig. 6 Frequency histograms of radioiodine uptake from areas in Udorn Province which is in the northeastern portion of Thailand, latitude 17°N. The land is flat and not too productive. Udorn town has a population of 15,000. Phon Ngan is situated about 25 km east of Udorn town on the main road.

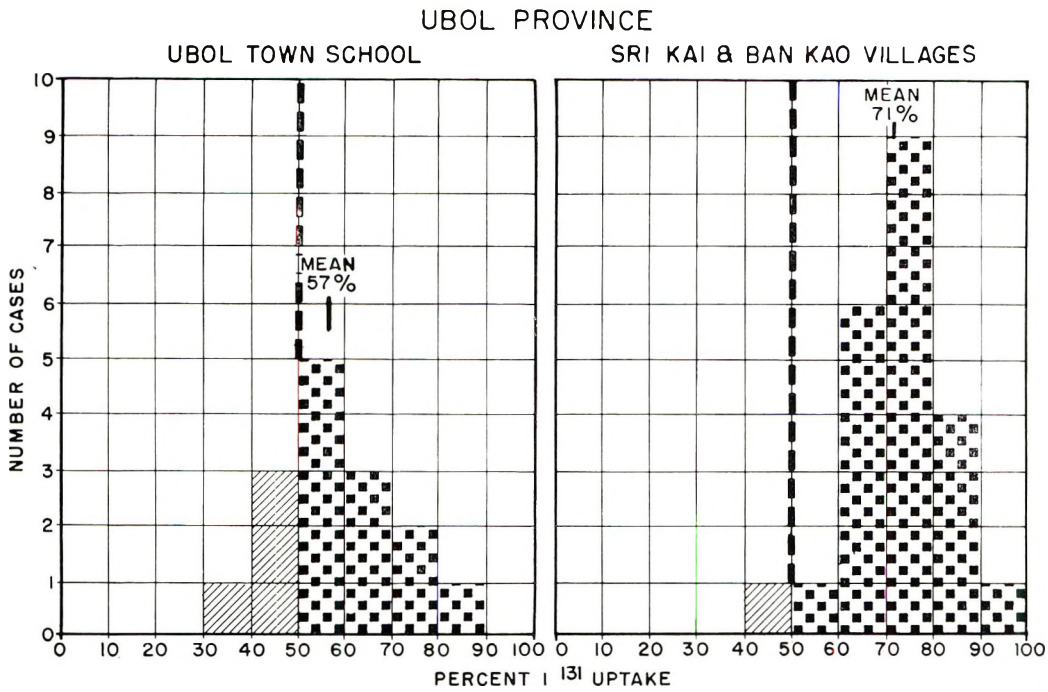

Fig. 7 Frequency histograms of radioiodine uptake from areas in Ubol Province which is located in the easternmost part of Thailand at latitude 15°N. Ubol town has a population of 38,000 but had just been partially destroyed by a serious fire. The villages of Ban Kao and Sri Kai are off the main roads and rather isolated.

TABLE 1

24-Hour I^{131} uptake by thyroid and iodine I^{127} excretion in schoolgirl groups

Site Province area	I^{131} uptake			I^{127} in urine/gm of creatinine			
	No. of subjects	Mean	SD	No. of subjects	Mean	SD	Mean I^{131} uptake this group
		%			μg		μg
Songkhla Province							
Bang Dan	10	40.1	± 6.7	8	74.5	±19.9	39.4
Haadyai	12	33.5	± 8.2	9	70.3	±20.5	33.4
Chieng Mai Province							
Chieng Mai	14	61.6	± 9.3	11	29.0	± 5.6	63.1
Hang Dong	13	63.9	±17.7	13	38.2	±22.5	63.9
Ban Fon	8	81.5	± 6.2	8	15.0	±12.3	81.5
Chieng Rai Province							
Ban Sam Gong	14	66.6	± 9.9	12	31.2	±24.5	67.7
Prae Province							
Huay Kae	12	78.0	±10.8	11	14.5	± 8.0	77.9
Udorn Province							
Udorn	15	41.9	±11.6	13	58.7	±29.5	41.8
Phon Ngam	12	59.7	± 9.5	10	40.5	±19.5	61.6
Ubol Province							
Ubol	15	57.4	±14.0	12	36.4	±18.5	56.7
Sri Kai and Ban Kao	10	76.9	± 9.5	10	17.4	± 6	76.9

μg of iodine per gm of creatinine. In table 2 are shown distributions of all groups of subjects with respect to levels of iodine excretion.

Figure 8 is a scattergram of all of these data with percentage uptake of radioactive iodine as the vertical axis and urinary excretion of iodine as the horizontal axis. The means of each area are plotted in similar fashion in figure 9.

The regression curvilinear shown in figure 9 was developed from the formula: $y=a-bx+cx^2$. The values for a, b, and c were developed from the raw data of uptake and excretion of iodine. The formula thus derived is as follows:

$$y=94.6949 - 1.119725x + 0.004588\ x^2$$

The differences between areas were tested for significance by the *t* test. The values obtained in the Songkhla area and Udorn town are significantly different from those in the rest of the country.

In table 3 are presented data derived from examinations of the thyroid by one observer on 2479 female and male school children, aged 7 to 18 years. These groups furnished the samples that were used for special studies of radioiodine uptake and stable iodine excretion. Goiter was virtually nonexistent in Songkhla Province (and Haadyai town) where uptakes and excretion ratios were observed to be normal. On the other hand, in all other areas the incidence of goiter was higher. Samples of children in these schools presented metabolic evidence of iodine malnutrition (tables 1 and 2).

Radioiodine uptake studies in military groups from 5 areas (fig. 10, table 4) appeared to indicate less evidence of iodine malnutrition than was noted in the civilian school girls. The mean uptake values in three sites: Songkhla, Bangkok and Ubol were below 50%. At Chieng Mai and Udorn the mean values were almost 60%. The ranges of values in the military tended to have a greater spread than those in the civilian groups. The mean values for stable iodine excretion are presented in table 4. With one exception, i.e., Songkhla Province, when the mean uptake values were below 50%, the mean iodine excretion was above 50 μg per gm of creatinine (Bangkok and Ubol); also, at Chieng Mai where the mean uptake was above 50% the excretion ratio was below 50. The reason for the low urinary iodine values at Songkhla is not clear. It will be recalled that the

TABLE 2

Urine concentration of I^{127} per gram of creatinine in schoolgirl group

Location	No. cases	0–50	51–100	101–150	151–200	201–250	251–300	301–350	351–400	Mean	SD
Songkhla Province											
Haadyai	9	1	8	–	–	–	–	–	–	70.3	±16.5
Bang Dan	8	2	6	–	–	–	–	–	–	74.5	±19.9
Chieng Mai Province											
Chieng Mai	11	11	–	–	–	–	–	–	–	38.9	±18.6
Hang Dong	13	9	4	–	–	–	–	–	–	38.2	±16.1
Ban Fon	8	8	–	–	–	–	–	–	–	16.6	±14.6
Chieng Rai Province											
Ban Sam Gong	12	10	2	–	–	–	–	–	–	31.2	±14.3
Prae Province											
Huay Kae	11	11	–	–	–	–	–	–	–	14.5	± 8.0
Udorn Province											
Udorn	12	5	7	–	–	–	–	–	–	58.7	±15.8
Phon Ngam	10	9	1	–	–	–	–	–	–	38.0	±13.5
Ubol Province											
Ubol	12	10	2	–	–	–	–	–	–	36.4	±15.3
Sri Kai and Ban Kao	10	10	–	–	–	–	–	–	–	20.4	±13.3
U.S.A.											
Washington, D. C. School	19	—	3	6	4	3	1	1	1	123.5	±27.7

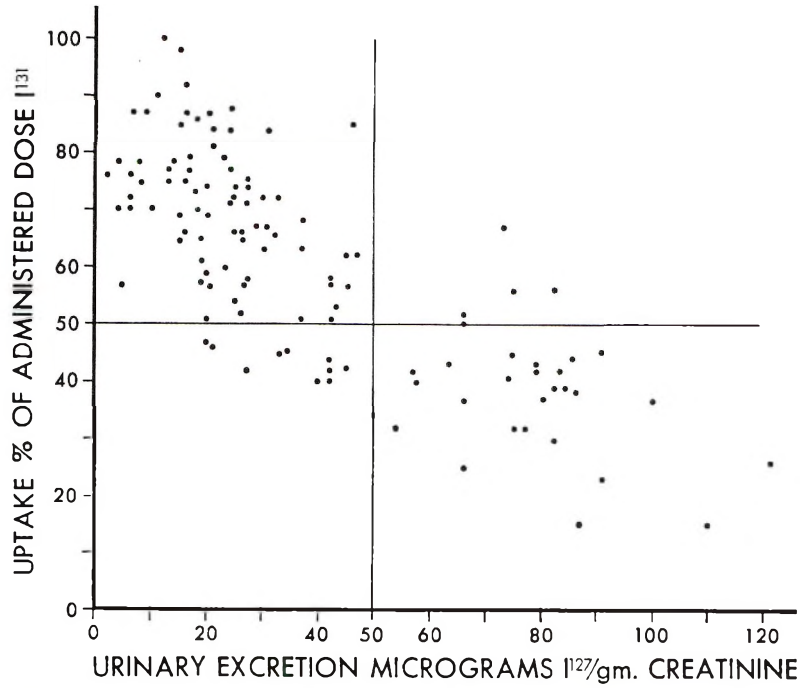

Fig. 8 The relation between uptake of radioactive iodine by the thyroid gland and the urinary excretion of stable iodine per gram of creatinine. Scattergram of determinations on all school girls studied.

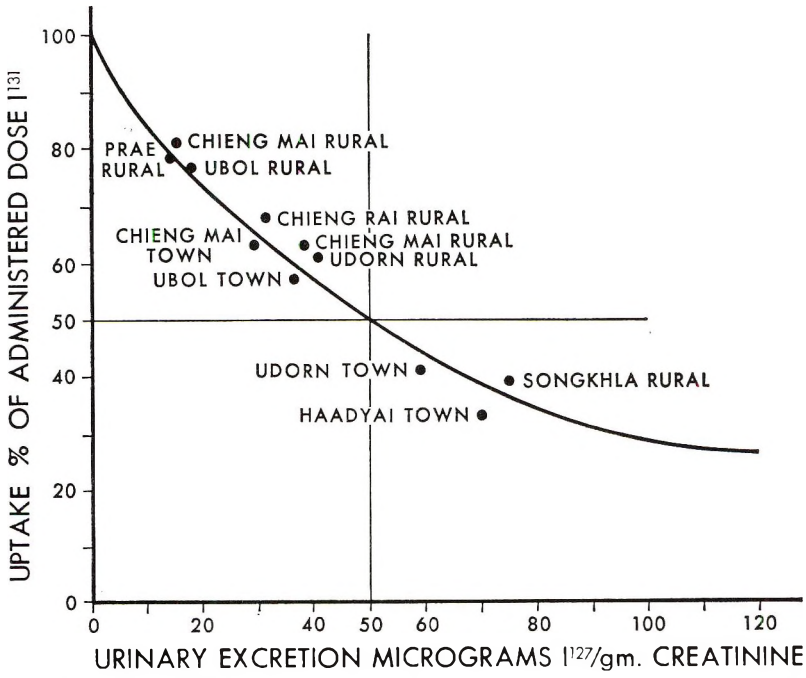

Fig. 9 The mean relations for each site studied between uptake of radioiodine by the thyroid gland and the urinary excretion of stable iodine per gram of creatinine. The calculation of the regression curve is explained in the text.

TABLE 3

Prevalence of goiter in school children

School location	No. examined	Age	Sex	Thyroid status[1]			
				N	1	2	3
Bang Dan	74	7–12	M	74	—	—	—
Songkhla Province	76	7–12	F	76	—	—	—
Lomboonkulganya Haadyai	251	14–16	F	249	2(0.8%)	—	—
Thidanukhro Haadyai	150	14–16	F	144	6(4%)	—	—
Dara Academy Chieng Mai	521	10–16	F	426	95(22%)	—	—
Prince Royal College	121	16–18	M	103	18(15%)	—	—
Chieng Mai	99	16–18	F	71	28(28%)	—	—
Hang Dong School	35	10–16	M	32	3(9%)	—	—
Chieng Mai Province	32	10–16	F	27	5(16%)	—	—
Mae Thai	37	10–15	M	28	9(25%)	—	—
Chieng Mai Province	30	10–15	F	24	16(53%)	—	—
Ban Sam Gong	57	7–14	M	22	34(60%)	1(2%)	—
Chieng Rai Province	43	7–14	F	5	35(81%)	3(7%)	—
Rajinuthit Udorn	388	11–17	F	219	168(43%)	—	—
Naree-Noo-Kul Ubol	565	10–15	F	383	182(32%)	—	—

[1] N indicates normal thyroid; 1, 2, and 3 indicate grades of enlargement.

TABLE 4

24-Hour I^{131} uptake by thyroid and I^{127} excretion in military groups

Site Province area	I^{131} uptake			I^{127} in urine/gm creatinine			
	No. of subjects	Mean	SD	No. of subjects	Mean	SD	Mean I^{131} uptake this group
		%			μg		μg
Songkhla	15	36	± 7.1	15	38	± 6.5	36.0
Bangkok	12	48	± 8.9	8	55	±32.5	47.4
Chieng Mai	14	58	±14.5	11	29	±13.8	57.3
Ubol	13	46	±11.5	12	78	±29.9	47.5
Udorn	15	59	± 7.2	—	—	—	—
Washington, D. C.	—	—	—	15	179.5	±57.7	—

school girls studied in this area had the highest mean excretions observed in Thailand. For comparison with these Thai military groups, the mean excretion value of stable iodine per gram of creatinine of a group of U. S. military males stationed in Washington, D. C., was 179.5 μg of iodine per gm of creatinine.

DISCUSSION

The causal relationship of iodine deficiency to endemic goiter has had a long and controversial history since the element was first introduced in 1820 as a therapeutic agent. In retrospect, it is obvious that the ensuing controversy was dependent on the fact that far too much attention was given to goiter prevalence and not enough to iodine nutrition. Marine and Williams ('08) showed clearly that the iodine content of the thyroid gland could be correlated with its anatomical structure. Furthermore, Marine and Kimball ('17) demonstrated the long-term beneficial effects

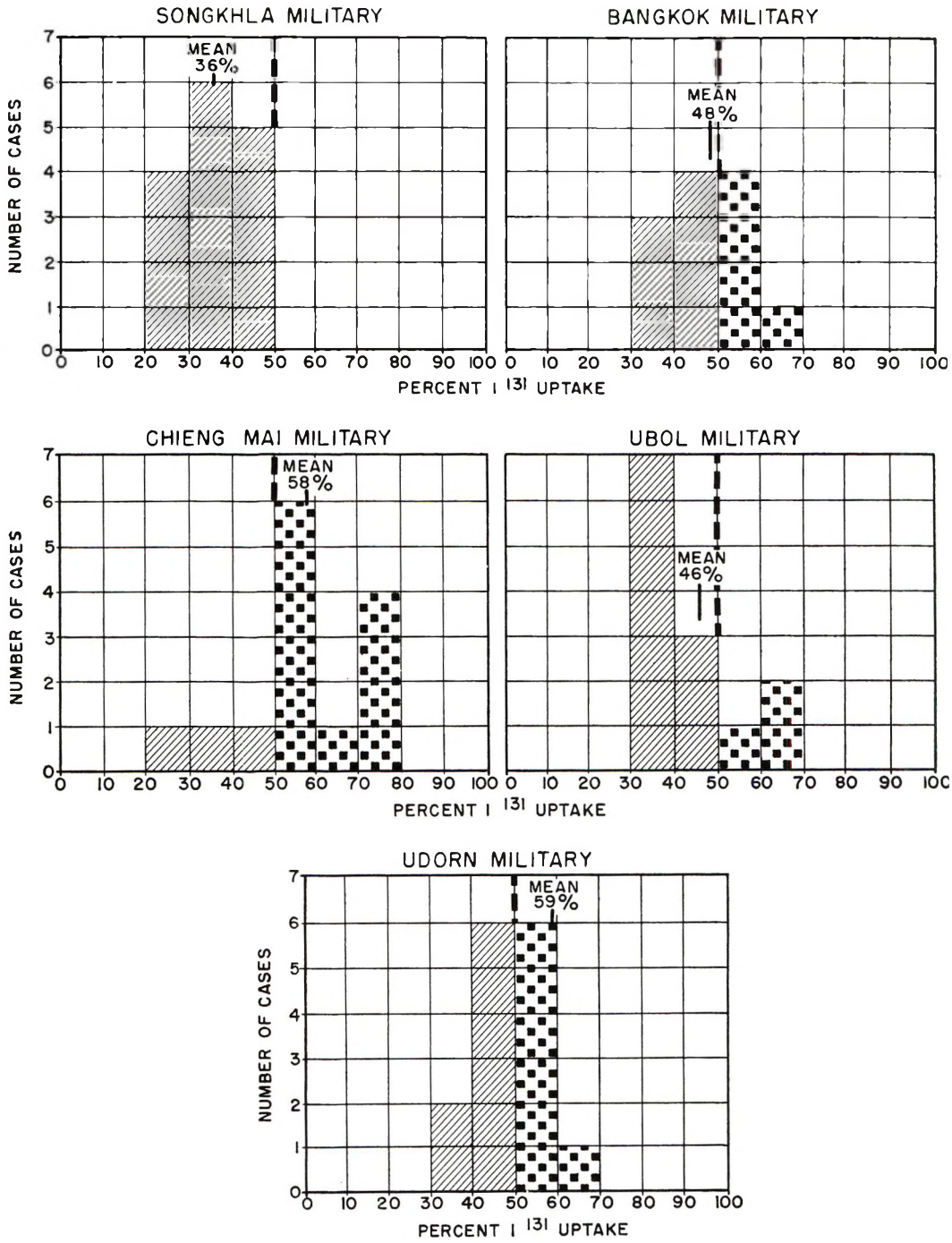

Fig. 10 Frequency histograms to show distribution pattern of radioiodine uptake in 5 military groups in various areas of the country.

of iodine on the prevention and treatment of endemic goiter in Akron, Ohio. Observations on iodine metabolism then became available in areas of endemic goiter (von Fellenberg, '26; Hercus et al., '25). Also, information began to be accumulated on the iodine content of soils, waters and foods from endemic and nonendemic areas (von Fellenberg, '26; Hercus and Roberts, '27; McClendon, '39).

Until recently, however, certain crucial data had yet to be presented. These are: (1) the experimental production of colloid goiter by an iodine-deficient diet, (2) an unbiased, blind study of the effects of iodine administration on goiter prevalence and (3) more complete observations on iodine metabolism in individuals with and without goiter in endemic areas in comparison with those from nonendemic areas. In recent years each of these critical points has been met. Colloid goiter has been produced experimentally in at least one species (Follis, '59) and is likely to be reported in others before long. A well-controlled study of iodine in the prevention of goiter in children has been reported from Guatemala and El Salvador (Scrimshaw et al., '53). Finally, during the past 10 years a number of specific studies of iodine metabolism in endemic goiter areas have been carried out in various parts of the world. These began with the now classical observations of the Andean Goiter Expedition, which was composed of workers from the United States and Argentina (Stanbury et al., '54). This group showed conclusively that the iodine content of the urine was reduced and that the uptake of radioactive iodine by the thyroid was increased in a group of euthyroid individuals with goiter. A summary of the data thus far reported by all observers will be found in table 5. It is clear that in areas where endemic goiter is prevalent an increased uptake of radioactive iodine by the thyroid is usually to be found. Moreover, and just as significant, a high uptake may be encountered in areas where goiter may not be prevalent. Since the largest proportion of dietary iodine is excreted by the kidneys, an even better index of iodine nutrition is the concentration of the element in the urine. From the data now available it is clear that iodine excretion in the urine is low in endemic areas. Furthermore, an inverse relationship may be demonstrated between radioiodine uptake by the thyroid gland and the excretion of stable iodine in the urine of a given individual.

The studies of iodine metabolism carried out in Thailand were designed to provide as much information as possible on the current status of iodine nutrition in various sections of the country among a highly sensitive group, adolescent females, who did not have goiter, or, at most, grade 1 enlargement. The results of radioactive iodine uptake in these civilian groups indicate that, except for three sites, two in Songkhla Province, which is located in the south on the penninsula, and one in Udorn in the northeast, the uptakes were well above normal, i.e., above 50%. These elevated values were observed at five sites in the northern provinces of Chieng Mai, Chieng Rai and Prae, at one site in Udorn Province, and at three sites in Ubol Province. Uptakes in military personnel in comparative locations tended to be lower although in two areas, Udorn and Chieng Mai, the mean values were above 50%.

The subject of iodine excretion is an important one which requires more detailed discussion. That ingested iodine is rapidly excreted in the urine has been recognized since the element began to be first used therapeutically for goiter and soon after for syphilis. In the early 1830's, Wallace (1836) was employing a starch test on the urine of patients he was treating for venereal disease by the iodide of potassium. Iodine balance studies had to wait some time, however. Not until 1923 were the first simultaneous estimations of intake and urinary excretion of the element reported (McClendon and Hathaway, '23). This report was followed by the observations of von Fellenberg ('23), Hercus et al. ('25), the more elegant balance studies of Scheffer ('33) and of Curtis and his associates ('43). As a result of the latter studies the *basal* daily requirement of the human adult was placed in the range of 44 to 75 µg, or an average of 67 µg per day. This value approximates 1 µg per kg of body weight per day. When these balance studies in normal subjects were supplemented by determinations of urinary iodine excretion in endemic and non-

endemic areas, the urinary excretion of iodine tended to be lower in persons living in goiterous regions than in those in which clinical goiter was not prevalent. The more recent results, summarized in table 5, indicate the same trend. The most extensive are those of Stanbury et al. ('54), who used 24-hour collection periods to establish their values.

In planning the studies reported herein, we were aware of the very great difficulties that would be encountered in the field if we attempted to make 24-hour collections. Hence, it was decided to apply a procedure that has been utilized for some time in nutrition surveys to evaluate vitamin excretion, i.e., to base the iodine content of casual urine samples on creatinine content of the same specimen. To our knowledge, this method of expression has not been applied heretofore. The correlation with the values of radioactive iodine uptake, which are shown in figures 8 and 9, would appear to make this method worthy of further study. The urinary excretion of iodine is the best criterion of iodine intake, even better than the uptake of isotopic iodine by the gland, since so many extraneous fac-

TABLE 5

Summary of studies of I^{131} uptake by the thyroid and I^{127} excretion

Author, year area	I^{131} uptake studies				I^{127} excretion in urine			
	No. cases	Goiter	Mean	Range or SD	No. cases	Goiter	Mean	Range or SD
			%				*µg/day*	
Stanbury ('54) Mendoza	103	+	58.6	11–89	25	+	23.6	
Eichorn ('55)	9	+	61	25–87				
Graz, Austria	33	0	57	14–90				
Roche ('56) Venezuelan Andes	14	+	50.4	12–79	12	+	22.5	9.0– 40.0
Roche ('57)	24	0	68	±21				
Venezuelan Andes	86	+	76	+10				
Lamberg ('58)					27	0	49.1	19.0– 87.9
Finland					102	+	41.2	6.0–119.3
Buzina ('59)		+	57.1			+	26.7	12.9– 44.6
Adriatic Sea		+	43.9					
		0	40.5					
Roche ('59) Kakuri, Venezuela	53	0	70.8					
Ferraris ('59) Sardinia	13	+	61.3	43–76				
Abu Haydar ('59)	13	0	5 > 50					
Lebanon	12	+	11 > 50					
Terpestra ('59) Holland	11	+	72.6	54–77	11	+	30.1	14–52
Kao ('60) Taiwan	88	+	56.5	15–92				
Diwany ('60) Egypt	72	0		18–55				
DeVisscher ('61) Congo	50	+	69.0	20–100	14	+	18.67	±6.9
Ramalingaswami ('61) India	40	+	69.9		11	+	10	
Romsai ('61) Thailand	50	+	74.3	45–90	18	+	8.61	3–33

tors may modify the intrathyroidal mechanisms of iodine metabolism.

Because no data were available for the urinary iodine-creatinine ratio in other population groups of similar ages, casual urine samples were obtained from 19 school girls from a parochial school in Washington, D. C. The mean value for this group was 123.5 μg of iodine per gm of creatinine, with a range from 71 to 375. This mean value is higher than any of those noted in Thailand. However, the Thai groups having the highest mean values, i.e., those in Songkhla Province, are within the lower limits of those of the Washington school girls. On the basis of such values as these, which are the only ones currently available, one may set 50 μg of stable iodine per gm of creatinine as a *tentative* lower limit of normal. It is hoped that these results will stimulate other groups to obtain comparative data. Such an approach, if valid, would be of inestimable value in assessing current iodine nutriture in population groups and would be a far more precise index of iodine malnutrition than is the prevalence of goiter.

Creatinine is another variable of real consequence which must be mentioned. It is generally accepted that the creatinine excretion per 24 hours is fairly constant for a given individual; the creatinine content of casual urine samples may fluctuate more. Moreover, the creatinine excretion is, of course, dependent on age and sex.

In conformity with the observations on iodine excretion in other endemic areas, summarized in table 5, the data presented herein would appear to indicate that a deficient iodine intake, and this alone, is the cause of goiter in those areas of Thailand surveyed in this study. There appears to be no reason to consider goitrogens, which, with certain exceptions have received much undue attention in recent years.

The factors responsible for the geographical variations in iodine uptake by the thyroid and excretion in the urine are not clear since data on the iodine content of soils, waters and foods are fragmentary. In the south, i.e., Songkhla Province, the inhabitants consume fresh seafood which would be expected to provide adequate iodine. Food diversification has not reached the stage in Thailand whereby seafood could supply the iodine needs of the rest of the country. It is of interest to note that iodine malnutrition appeared to be more severe in villages than in town populations surveyed. There is, of course, no salt iodization program nor any dairy industry, two probable sources of dietary iodine in the United States. It must be pointed out, however, that no current data are available on the sources and amounts of iodine consumed in the United States or in any other country.

In general, the prevalence of goiter paralleled the severity of iodine malnutrition among the school girls studied.

SUMMARY

A survey of iodine metabolism in nongoitrous school girls and military males was conducted in 7 areas of Thailand. Measurement of radioiodine uptake by the thyroid gland and determinations of stable iodine concentrations of casual urine specimens, expressed in terms of creatinine content, indicate that iodine malnutrition is prevalent in the northern provinces of Chieng Mai, Chieng Rai and Prae as well as in the eastern provinces of Udorn and Ubol. These observations are correlated with the prevalence of goiter in these areas.

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Nutrition of Salmonoid Fishes

X. QUANTITATIVE THREONINE REQUIREMENTS OF CHINOOK SALMON AT TWO WATER TEMPERATURES[1,2,3]

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Young chinook salmon (*Oncorhynchus tschawytscha*) in their fresh water feeding environment have been reported unable to synthesize 10 indispensable amino acids (Halver et al., '57). The present study was undertaken to determine the minimal requirement of one of these amino acids, threonine, under varying experimental conditions. Because fish are poikilothermous animals and the minimal protein requirement has been shown to be dependent upon the water temperature (De Long et al., '58), it was desirable to include the temperature variable in the measurement of at least one amino acid requirement. Experimental facilities with constant water temperature of 8°C were available at the Western Fish Nutrition Laboratory, Cook, Washington and 15°C at the experimental laboratory facilities at the Hagerman National Fish Hatchery, Hagerman, Idaho. Salmon fingerling diets were also available which contained purified proteins supplemented with crystalline L-amino acids for the nitrogen component of the ration and which allowed positive experimental control over the other nutrient components (Halver, '57a,b). Crude protein requirements (whole-egg protein) of approximately 40% of the diet have been described for salmon fingerlings held in water temperatures of 8°C (De Long et al., '58), and this crude protein content of the diet was maintained throughout each nutritional treatment at the two temperatures (8° and 15°C).

EXPERIMENTAL

In table 1 are listed the ingredients of the basal ration used in the three feeding experiments. The levels of protein, casein and gelatin were adjusted to provide a low level of threonine in the basal diet (see table 2). The limiting level of threonine had previously been approximated by preliminary feeding trials. In each instance, the whole proteins were supplemented with L-amino acids to give a crude protein content (N × 6.25) of 40 gm of dry diet. The essential amino acids were supplied in the balance and in the amounts found in 42 gm of whole-egg protein in experiment 1 and in 40 gm of whole-egg protein in

TABLE 1
Basal diet for threonine studies

	Exp. 1	Exp. 2, 3
	gm/200 gm of diet	
Casein	8.00	13.30
Gelatin	2.00	3.00
Amino acid mixture[1]	34.58	27.45
White dextrin	21.42	26.05
α-Cellulose	10.00	5.00
Corn oil	5.00	5.00
Cod liver oil[2]	2.00	2.00
Minerals[3]	4.00	4.00
CMC[4]	10.00	10.00
Vitamins[3]	3.00	3.00
Water	100.00	100.00

[1] Individual amino acids listed in table 2.
[2] USP Standard, Nutritional Biochemicals Corp., Cleveland.
[3] Mineral mix and vitamin mix contained the same ingredients as reported previously (Halver and Shanks, '60).
[4] Carboxymethylcellulose.

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[1] The experimental data in this paper are taken from a thesis submitted by Donald C. DeLong in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry, Purdue University.
[2] Presented at the meetings of the American Institute of Nutrition, Philadelphia, Pennsylvania, April, 1958.
[3] Purdue University Agricultural Experiment Station Journal Paper no. 1800.
[4] Present address: A. E. Staley Manufacturing Company, Decatur, Illinois.

TABLE 2

Amino acid content of threonine diets[1]

	AA[2] supplied by whole-egg protein		AA supplied by casein		AA supplied by gelatin		Total AA supplied by whole protein		AA supplied by crystalline AA		Protein supplied by crystalline AA	
	42%	40%	8%	13.3%	2%	3%	Exp. 1	Exp. 2, 3	Exp. 1	Exp. 2, 3	Exp. 1	Exp. 2, 3
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Arginine·HCl	2.66	2.5	0.31	0.51	0.16	0.24	0.47	0.75	2.19	1.75	4.53	3.51
Histidine·H_2O·HCl	0.86	0.8	0.24	0.39	0.02	0.03	0.26	0.42	0.60	0.38	1.01	0.63
Isoleucine	3.31	3.2	0.56	0.90	0.04	0.06	0.59	0.96	2.72	2.24	1.81	1.49
Leucine	3.78	3.7	0.72	1.20	0.07	0.10	0.79	1.30	2.99	2.50	1.99	1.60
Lysine·HCl	2.96	2.9	0.62	1.02	0.13	0.20	0.75	1.22	2.21	1.68	2.64	2.01
Methionine	1.72	1.6	0.28	0.46	0.02	0.03	0.30	0.49	1.42	1.11	0.83	0.67
Phenylalanine	2.60	2.5	0.46	0.76	0.04	0.06	0.50	0.82	2.10	1.68	1.11	0.89
Threonine	1.81	1.7	0.32	0.54	0.04	0.06	0.36	0.60	varied			
Tryptophan	0.62	0.6	0.09	0.15	0.00	0.00	0.09	0.15	0.53	0.45	0.45	0.38
Valine	3.05	2.9	0.56	0.93	0.06	0.09	0.62	1.02	2.39	1.88	1.79	1.40
Glycine									5.00	5.00	5.83	5.83
Glutamic acid									3.24	2.50	1.74	1.49
Aspartic acid									2.60	2.60	1.70	1.70
Alanine									2.07	1.18	2.04	1.16
Proline									2.95	2.95	2.24	2.24

[1] Grams per 100 gm of dry diet. [2] AA indicates amino acids.

experiments 2 and 3, except for the variable, threonine. The threonine content of casein and gelatin was determined by microbiological assays using the media of Steele et al. ('49), and the hydrolysis procedure described by Singleton.[5] Higher levels of threonine were obtained by adding crystalline L-threonine to the basal ration at the expense of an equal amount of nitrogen included as L-alanine, in experiment 1 and as L-proline in experiments 2 and 3. The methods of diet preparation and general feeding techniques were the same as those previously reported for carefully controlled salmon fingerling diet trials (Halver, '57a). Experiments 1 and 2 were conducted at the experimental laboratory facilities located at the Hagerman National Fish Hatchery, Hagerman, Idaho and experiment 3 was conducted under cooler water conditions (8°C) at the Western Fish Nutrition Laboratory, Cook, Washington. Actively feeding chinook salmon fingerlings were divided into lots of 200 fish each, one lot per trough with average weights of individuals 1.25 (exp. 1), 3.00 (exp. 3), and 5.90 gm (exp. 2), respectively, depending upon the age of the fish used for the different experiments. Weight variation between lots in any one trial was minor (see table 3). In each instance, the fish were gradually adapted during a one-week period to the experimental environment and to the 40% crude protein diet used in these quantitation studies (1.7% of threonine). Fish were fed three times

TABLE 3

Growth data from threonine quantitation studies

Threonine in diet	Starting average weight	Final weight	Individual mortality	Total average gain
%	*gm*	*gm*		*gm*
Experiment 1 at 15°C				
0.40	5.83	5.61	15	−0.22
0.50	6.00	6.70	22	0.70
0.60	5.93	7.35	11	1.42
0.80	5.99	8.03	20	2.04
1.00	5.97	9.76	13	3.79
1.81	6.25	9.53	11	3.28
Experiment 2 at 15°C				
0.60	1.26	3.07	6	1.81
0.80	1.26	3.40	7	2.14
0.90	1.22	3.81	4	2.59
1.00	1.26	3.50	6	2.24
1.10	1.29	3.29	7	2.10
1.20	1.28	3.53	4	2.25
1.80	1.22	3.51	2	2.29
Experiment 3 at 8°C				
0.60	3.02	5.39	6	2.37
0.70	3.12	5.88	12	2.76
0.80	3.06	6.19	49	3.13
0.90	3.09	6.43	4	3.24
1.00	3.05	6.36	8	3.31
1.10	3.00	6.38	6	3.38

[5] Singleton, V. L. 1951 Ph.D. thesis, Purdue University.

daily, 6 days weekly on a rigid schedule by one individual. Dead fish were removed as soon as observed, troughs were cleaned daily and disinfected and cleaned thoroughly during the weighing periods. The fish were weighed bi-weekly during the 10-week experimental period using the techniques previously described for vitamin and amino acid studies with salmon (Halver, '57a,b). Equipment common to more than one trough was disinfected between troughs to minimize inadvertent transfer of food particles or disease organisms. After completion of each experiment, the fish were examined closely for any pathological changes by an histologist[6] and samples were prepared at this time for subsequent proximate analysis using the techniques described by Wood et al. ('57).

RESULTS

Growth data for the 10-week experimental period are summarized in table 3. Fish fed the 0.4% L-threonine diet showed a loss in weight. With progressively higher levels of L-threonine, however, the growth increased to a maximum at a level of threonine corresponding to 1.0% of the dry ration. When these data were applied to graph-plot type calculations of the requirement as seen in figure 1, however, a requirement of approximately 0.9% threonine of the dry ration was obtained. In the second experiment, at 15°C, a smaller range of threonine values, clustered near those previously obtained for maximal growth in the first experiment, was used. The optimal growth under these conditions was obtained at 0.9% of L-threonine which supported the results of the first trial. These data were also plotted on the graph-plot in figure 1. In order to extend the temperature range and study the effect of threonine at cooler water temperatures, the third experiment was carried out at 8°C within a relatively narrow range of threonine intake with 1.0% of threonine in the diet as the median level fed. Once again a requirement of approximately 0.9% of threonine was indicated for optimal growth under these experimental con-

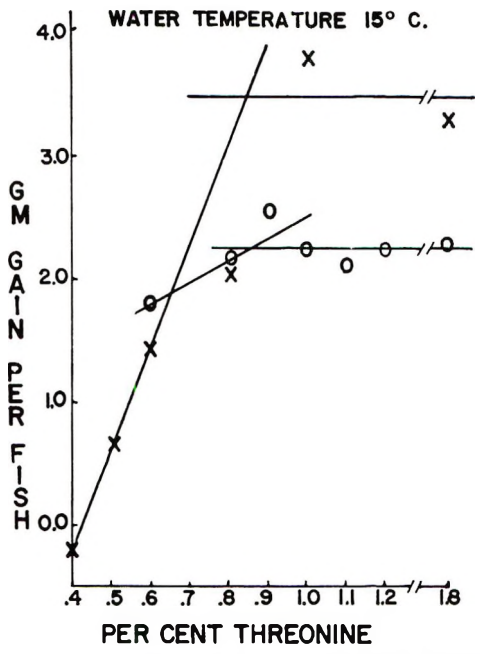

Fig.1 Theonine requirement at 15°C. Growth response of chinook salmon fingerling fed different levels of L-threonine. Lower curve shows data with larger fish in experiment 1 and upper curve summarizes data of experiment 2 with small fish.

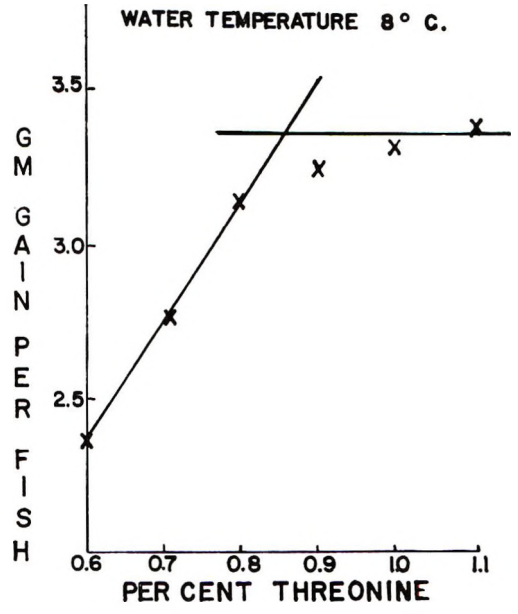

Fig. 2 Threonine requirement at 8°C. Summary of growth response of chinook salmon fingerling fed L-threonine at levels indicated in experiment 3. Inflection point indicates L-threonine requirement consistent with response observed at 15°C.

[6] W. T. Yasutake, present address: Western Fish Disease Laboratory, Seattle, Washington.

ditions. The high mortality rate observed at the 0.8% threonine level was due to an accident which occurred while weighing these fish during the 6th week of the feeding trial. After termination of the various feeding trials, careful observations for gross pathology and for changes in the proximate analysis of the entire fish showed no statistical differences in these areas between the groups of fish given these nutritional treatments.

DISCUSSION

The growth data suggest that the threonine requirement of chinook salmon at 8° and 15°C is approximately 0.9% of the dry diet. This constant requirement, in direct contrast with the minimal protein requirement which varied with water temperatures, was obtained with diets containing about 40% of crude protein indicating a threonine requirement of about 2.25% of the dietary protein. Similar results have been obtained with the rat which requires approximately 0.5% of threonine at a crude protein level of 16% or a threonine requirement of about 3.1% of the dietary protein (Frost, '50). The weanling pig requires 0.4% of threonine with a diet containing 13.2% of protein or 3.0% of the dietary protein (Beeson et al., '53). The chick requires 0.6% of threonine with a diet containing 20% of protein, again indicating a requirement of 3.0% of the dietary protein (Block and Weiss, '56). When based on the percentage of dietary protein, the requirement for the rat, pig and chick is higher than that of the salmon in their fresh water feeding cycle. These data then seem to indicate that although the protein requirement of the salmon is considerably higher during its fresh-water feeding period, the actual threonine requirement, when calculated on the basis of dietary protein, remains in the same order of magnitude or perhaps slightly lower than that of other experimental animals.

In these nutrition trials, the protein intake was maintained constant at that level previously found adequate for chinook salmon in the lower water temperature (8°C). This same level of protein, even if adequately balanced, could not be expected to satisfy the crude protein demands for maximal growth at the higher water temperature (15°C) (De Long et al., '58). Therefore the threonine requirement of young salmon might seem consistent at 0.9% of the dry diet when 40% of whole-egg protein was fed but could shift upwards in warmer waters when the crude protein requirements were satisfied by feeding higher protein diets (50% at 15°C). Whether the threonine requirement is constant at different protein intakes, since it is constant at different water temperatures with the same protein intake, is a problem worthy of future study.

SUMMARY

The threonine requirement of chinook salmon has been investigated in three separate feeding trials at 8° and at 15°C. The threonine requirement of chinook salmon fed 40% of crude protein diets remained consistent at approximately 0.9% of the dry ration.

ACKNOWLEDGMENT

The feeding trials were conducted by Robert Smith and Edward Larson at the Hagerman experimental laboratory facilities located at the Hagerman Fish Hatchery, Hagerman, Idaho and by Roy Hales at the Western Fish Nutrition Laboratory, Cook, Washington.

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Use of Radioyttrium to Study Food Movement in the Small Intestine of the Rat[1,2]

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Until relatively recently, techniques for studying the passage of materials through the gut have included the use of glass beads, small seeds, dyes, and inert indicators such as carbon, chromic oxide, or barium sulfate (Hoelzel, '30). The data produced by these methods were often misleading because these indicators can have a variety of undesirable effects (Alvarez, '48); the beads and seeds can become separated from the food material during alimentary passage, dye indicators may not be accurately located, and $BaSO_4$ has laxative characteristics.

A radioactive tracer technique could be very useful because the isotope measuring methods are relatively easy to carry out with precision, and the radioisotopes can be given to the animal without increasing the bulk of the diet or producing undesirable side effects. The use of a nonabsorbable radionuclide, Y^{91}, to study the passage of material through the rat digestive tract is described in this paper.

METHODS

Thirty-two male albino rats[3] weighing from 100 to 115 gm and previously maintained with a 1.9% calcium diet[4] were used for the solid dose experiments. They were trained over an 8-day period to eat a small meal quickly, and on the 9th day were fasted. On the 10th day each rat was given 2 gm of ground feed[5] mixed with 1 ml of an acidified water solution which contained 6.62 mg of $CaCl_2 \cdot 2H_2O$ and 1 μc of Y^{91}. The rats were allowed to eat for 10 minutes, during which time the average rat ate 80% of the food. The animals received no more food or water, and from two to 5 rats were killed after time periods of 30 minutes, 1, 2, 3, 4, 6, 8, and 12 hours. Immediately after the death of a rat, the abdomen was opened and the alimentary tract clamped off directly above and below the stomach and above the cecum. The entire tract was then removed and divided into 6 segments: segment 1, stomach (length, 2.5 cm); segment 2, duodenum (length, 21 cm); segment 3, jejunum (length, 21 cm); segment 4, jejunum — possibly some ileum (length, 21 cm); segment 5, ileum (length, 21 cm); segment 6, cecum plus colon (length, 13 cm). The segments were placed in test tubes and then counted in a well scintillation counter. The uneaten dose was wet-ashed with concentrated HNO_3 and then counted so that the actual dose given to each rat could be calculated.

Coprophagy was not a problem because the animals were fasted for 24 hours before being given the test materials and the experiment was terminated before the animals could excrete pellets containing Y^{91}.

The experiment was repeated using similar rats fed a liquid dose. Forty-one rats were permitted water but no feed for 18 hours prior to the administration of the isotope. The rats were lightly anesthetized with ether and given 1 ml of dosing solution by gavage. The animals were deprived of further food and water, and from three to 6 rats were killed after time periods of 30 minutes, 1, 2, 3, 4, 6, 8 and 12 hours. Sampling and counting procedures were identical to those used for the solid dosed rats. The data were calculated as percentage of administered dose recovered per section of digestive tract.

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[2] Supported in part by contract AT30-1-2147 from the Atomic Energy Commission.

[3] Carworth Farms, New City (Rockland County), New York.

[4] Rockland, Rockland Farms, New City (Rockland County), New York.

[5] See footnote 4.

RESULTS AND DISCUSSION

In order for a radioisotope to be useful as a marker it must remain with the food and be nonabsorbable. To establish this for Y^{91}, an *in vitro* experiment was performed in which finely divided rat food was labeled with Y^{91}, covered with an excess of water and then made into a suspension by shaking. The suspension was titrated with either NaOH or HCl to achieve a desired pH in the range from 3.0 to 8.0 pH units. After one hour the samples were centrifuged and the solid and supernatant counted separately. The results showed that at pH3 about 85% of the Y^{91} stayed combined with the ground rat food and as the pH increased, about 96% of the Y^{91} was combined with the food at pH8. Kidneys of rats given Y^{91} orally were collected and none counted significantly above background. In addition, the total recovery of Y^{91} in the digestive tracts of the rats used for these studies averaged $97.9 \pm 0.8\%$. Thus Y^{91} does not undergo absorption and by staying with the food is a valid indicator of food location.

The data obtained from the experiments following the passage of a meal of solid or liquid marked with Y^{91} is shown in table 1. A calculation called "relative per cent dose" has been used in presenting the data, and is defined as follows:

$$\text{Relative \% dose for segment S} = \frac{\text{\% dose recovered in segment S}}{\text{\% dose recovered in whole gut}} \times 100.$$

The total relative per cent dose of each animal is the sum of each of 6 segmental relative per cent doses, and is always equal to 100%. Since Y^{91} is not absorbed by rat gut, a relative per cent dose calculation is almost the same as the absolute or observed dose present. The calculation was made to remove small deviations because recovery was usually not 100%. The relative per cent doses appearing in the 6 segments for the various time groups shown in table 1 contrast the passage of liquid and solid materials. Statistics using the nonparametric Wilcoxon paired test (Snedecor, '56) indicated that the liquid dose left the stomach at a faster rate than the solid dose ($P < 0.01$). There was no significant difference between the initial appearance of liquid dose in the cecum and the initial appearance of solid dose in the cecum. Once food began entering the cecum, however, the rate of filling was faster in the liquid-dosed rats. These observations may be explained by postulating that the first of the solid dose to pass out of the stomach does so after the same time period as that required for the first of the liquid dose to leave the stomach. Both food fronts move at approximately the same rates through the small gut, and reach the cecum at about the same time. But since the rate of solid food leaving the stomach is slower than the rate characteristic of liquid food, the rate of cecal filling is slower in solid-dosed rats. This explanation does not exclude the possibility that the rate of movement of food in a particular part of the small intestine changes either with time, with the amount of food present, or with both. If it is assumed that the initial solid and liquid food fronts move at about the same rates, then the data provide evidence for just such a change in rate, because in calculations (see following paragraph) indicate that the rate of movement of solid food in segment 4 at a particular period is considerably less than the corresponding rate for liquid food.

The Y^{91} data permit calculation of an estimate of the rate of food transport through a segment. In order to do this the

TABLE 1

Y^{91} content of the various segments of the rat intestine expressed as relative per cent dose[1]

Time group	No. of rats	Segment 1	2	3	4	5	6
hours							
		Solid-dosed rats					
0.5	5	70	2	8	15	5	0
1	4	38	5	20	24	14	0
2	5	41	2	8	16	20	14
3	5	11	1	4	16	34	33
4	4	8	1	7	17	26	39
6	5	2	0	1	3	12	82
8	2	2	0	1	0	1	95
12	2	0	0	0	0	0	100
		Liquid-dosed rats					
0.5	5	58	3	13	24	1	0
1	6	14	5	32	33	16	0
2	6	7	1	2	32	57	0
3	5	8	1	2	12	38	40
4	6	6	1	2	6	25	60
6	6	2	0	1	2	18	77
8	3	0	0	0	0	0	99
12	3	0	0	0	0	1	98

[1] Data are for liquid and solid content.

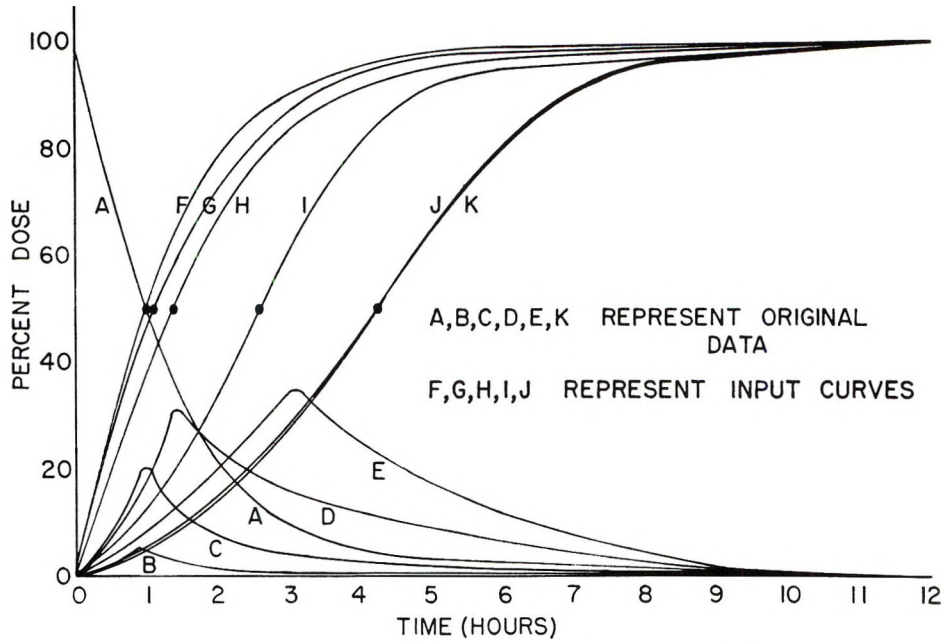

Fig. 1 A indicates observed data and represents segment 1 output; B, C, D, and E indicate observed data and represent the combined effects of input and outgo for segments 2, 3, 4, and 5, respectively; K indicates observed data and represents cecum input; F is the inverse of A and represents input to segment 2; G shows output from segment 2 and the segment 3 input; H, segment 3 output and segment 4 input; I, segment 4 output and segment 5 input; J, segment 5 output and segment 6 input (calculated value). The points indicated on the graph represent segmental transit half-times.

relative per cent dose recovered at each time period was plotted for each gut segment (the bell-shaped curve of fig. 1). These curves are resultants of two processes — input into a segment and outgo from that segment. For a nonabsorbable material, the output from a segment is necessarily the input to the next adjoining segment. Thus, the input to segment 2 (duodenum) is equal to the rate at which material left segment 1 (stomach). The output from segment 2 can be calculated as equal to the input from the preceding segment minus the resultant. This, then, is the input to segment 3. The input curves of segments 2 to 5 were calculated in this manner, and are sigmoid curves shown in figure 1. The original data for the 6th segment represents input since no Y^{91} was excreted; this serves as a check on the accuracy of these calculations since the actual data agreed well with the calculated input (curves J and K of fig. 1).

The data of the solid dose experiment only are shown; curves for the liquid dose were essentially similar to those of the solid dose experiment.

When the input curves had been constructed for each of the segments it was possible then to determine segmental transit half-times from the ordinates of the graphs. This value is the time at which one-half of the dose has left the segment (input to one segment equals output from preceding segment). The time between half-times of consecutive segments can also be read from the graphs, and since the length of each segment was known (see Methods section), the transport rate in centimeters per hour may be calculated.

The results of these calculations are shown in table 2 for both solid and liquid dose experiments. The highest transport rates for each type of dose occurred in the proximal part of the small gut; these rates diminished continuously until, in the distal part of the small intestine, they had decreased by a factor of almost 20. This is in agreement with observations of Cramer and Copp ('59), who reported that the

TABLE 2

Transport rate per segment and segmental transit half-times for liquid and solid material in the rat intestine

	Segment				
	1 (stomach)	2 (duodenum)	3 (jejunum)	4 (jejunum, possibly some ileum)	5 (ileum)
Solid					
Transport rate		220 cm/hr	73 cm/hr	18 cm/hr	13 cm/hr
Transit half-time (T ½)	60 min	6 min	18 min	72 min	102 min
Liquid					
Transport rate		210 cm/hr	70 cm/hr	55 cm/hr	11 cm/hr
Transit half-time (T ½)	36 min	6 min	18 min	24 min	120 min

transit was faster in the duodenum and jejunum. The time at which one-half the solid dose had entered the cecum was 21% greater than the corresponding time for the liquid dose, and appeared to be due to a slower rate of movement of solid material through the stomach and 4th segment (distal jejunum). Both solid and liquid doses exhibited essentially the same transit rates in the other parts of the alimentary tract.

This nonabsorbable radio-indicator technique has several important benefits. First, it permits *in vivo* experiments with undisturbed alimentary tracts, preserving the natural physiological conditions. Second, both liquid and solid food may be used, thus paralleling a normal diet. Third, the method seems quite reliable because the actual data agree with calculated values (fig. 1). Fourth, the technique is simpler and more accurate than an experimental technique in which the whole gut is moved under a collimated GM tube (Cramer and Copp, '59).

The technique should prove useful for determining the effects of nutrients, drugs and inorganic salts on the transport of food through the gut, and may also be used for absorption studies when combined with one or more absorbable radioisotopes.

SUMMARY

Nonabsorbable yttrium-91 was used to label solid and liquid food which, when fed to rats, made it possible to calculate the rate of passage of food through the small intestine. To accomplish this, rats were killed at predetermined time intervals after feeding, and the percentage of the Y^{91} dose present in a particular portion of the digestive tract was plotted against time for each of the consecutive alimentary segments; each curve was then resolved into input and outgo components. The highest rates of passage occurred in the proximal small gut; the rates then diminished continuously until, in the distal small intestine, they had decreased by a factor of almost 20. Solid material left the stomach at a slower rate than liquid, but the time necessary for material to first appear in the cecum was about three hours for both liquid- and solid-dosed rats. The time at which one-half of the solid entered the cecum was 21% greater than the corresponding time for liquid, and appeared to be due to a slower rate of movement of solid through the stomach and distal jejunum. Both solid and liquid doses exhibited essentially the same passage rates in the other portions of the small intestine.

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Effect of Lime Treatment of Corn on the Availability of Niacin for Cats[1,2]

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Several reports in the literature on the improved availability of niacin resulting from lime treatment of corn have suggested that the niacin is released from its "bound" form in corn by the lime treatment (Kodicek et al., '56; Kodicek, '60) or that the proportions of the amino acids of corn absorbed are improved. The latter is suggested by the observation that zein is less soluble after the corn has been lime-treated (Bressani et al., '58). The niacin in corn has been reported to be partially unavailable for the rat (Kodicek, '60), chick (Coates et al., '52) and pig (Kodicek et al., '56). The results of Krehl et al. ('44), however, suggest that the dog is able to utilize the "bound" niacin present in cereal products.

To study further the niacin availability of corn and lime-treated corn, the excretion of N-methyl nicotinamide in the urine of cats was used as a measure of the availability of niacin to these animals. The cat was chosen as the experimental animal because this species cannot convert tryptophan into niacin (Carvalho da Silva et al., '52).

MATERIALS AND METHODS

Growing cats, weighing between 800 and 900 gm, were placed in individual metabolism cages and fed the following diet in grams per 100 gm: casein, 8.0; gelatin, 15.0; DL-methionine, 0.2; fat,[3] 18.0; salt mixture (Hegsted et al., '41), 4.0; cod liver oil, 2.5; either corn or lime-treated corn, 52.0, and 10 ml of a vitamin solution containing in milligrams: thiamine, 2; riboflavin, 4; pyridoxine, 2; menadione, 3; niacin, 16; pantothenic acid, 16; inositol, 40; folic acid, 0.1; biotin, 0.1; *p*-aminobenzoic acid, 16, and choline chloride, 400. The lime treatment of corn was carried out according to the procedure described elsewhere (Bressani et al., '58). Twenty grams of the ration were suspended in water to make a soft gruel which was fed to the animals twice a day, between 8 and 9 AM and between 3 and 4 PM. All animals consumed all of the food offered within one hour. Water was given ad libitum.

During three experiments, 4 cats were fed the raw corn diet and 4 others the lime-treated corn diet. In the first experiment, the two diets were supplemented with both niacin and tryptophan at 16 mg and 0.20 gm in the DL- form per 100 gm of ration, respectively. In the second experiment only niacin was used; and in the third experiment only tryptophan was added. The diets were fed for an adaptation period of 4 days and then for two experimental periods of 4 days each during which urine and feces were collected. The diets were then reversed so that the animals that had received the raw corn diet were fed the lime-treated corn diet and vice versa. All animals were weighed at the beginning and end of every balance period.

On the 4th day of each experimental and adaptation period, an aliquot of urine was taken from each sample for the determination of N-methyl nicotinamide and creatinine by the fluorometric (Huff et al., '47) and the picric acid (Clark et al., '49) methods, respectively. Nitrogen was determined in the feces, urine, and food by the macro-Kjeldahl procedure using selenous acid as catalyst. Niacin in the rations was determined by the microbiological method (USP, '50).

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[1] This investigation was supported by grant-in-aid from the Williams Waterman Fund.
[2] INCAP Publication I-223.
[3] Crisco, Procter and Gamble, Cincinnati, Ohio.

RESULTS

In table 1 are shown the results obtained from the treatments described. In experiment 1, where both tryptophan and niacin were added, the average niacin intake was 5.0 mg per cat per day for both the raw and lime-treated corn diets. The average 24-hour excretion of N-methyl nicotinamide was 4.53 for the raw corn and 4.28 mg for lime-treated corn. The N-methyl nicotinamide excretion expressed as milligrams per milligram of creatinine was 0.26 and 0.27 mg for the raw corn and lime-treated corn diets, respectively. The difference in N-methyl nicotinamide excretion between the two was not statistically significant.

In experiment 2, where only niacin was added to the diet, the N-methyl nicotinamide excretion was 5.04 and 4.61 mg for the raw and the lime-treated corn diets, respectively, and 0.20 and 0.18 mg expressed as milligrams of N-methyl nicotinamide per milligram of creatinine. The niacin intakes for experiment 2 (see table 1) were slightly higher than in experiment 1. Niacin contamination of a new stock of the vitamin solution probably accounts for the slightly higher N-methyl nicotinamide values obtained. The differences between raw and lime-treated corn in experiment 2 and between experiment 1 and 2 were not statistically significant.

In experiment 3, where tryptophan was added to the diets but niacin was withdrawn, the niacin intakes were 0.9 and 0.6 mg per day for the corn and lime-treated corn diets, respectively, and the N-methyl nicotinamide excretions were 0.36 and 0.31 mg per day. This difference was not statistically significant. The excretion expressed as milligrams per milligram of creatinine was the same for both diets.

The nitrogen balance data are shown in table 2. When tryptophan was withdrawn from the diet, there was a decrease in nitrogen retention whether the diet contained raw or lime-treated corn. Nitrogen balance was restored to initial values by the addition of tryptophan to the diet. Presence or absence of niacin had no effect on nitrogen balance, and nitrogen absorption was not

TABLE 1

Urinary N-methyl nicotinamide excretion of cats fed corn or lime-treated corn diets

Experiment no.	Treatment[1]	Niacin intake	N-methyl nicotinamide	Creatinine	N-methyl nicotinamide/mg creatinine
		mg/day	*mg/day*	*mg/day*	*mg*
1	Corn + T + N	5.0	4.53 ± 1.18[2]	19.02 ± 0.82	0.26 ± 0.08
	Lime-treated corn + T + N	5.0	4.28 ± 1.05	18.18 ± 0.84	0.27 ± 0.07
2	Corn + N	6.0	5.04 ± 1.27	27.38 ± 0.96	0.20 ± 0.06
	Lime-treated corn + N	6.0	4.61 ± 1.00	28.96 ± 1.24	0.18 ± 0.05
3	Corn + T	0.9	0.36 ± 0.24	29.97 ± 1.05	0.01 ± 0.01
	Lime-treated corn + T	0.6	0.31 ± 0.13	31.93 ± 1.47	0.01 ± 0.01

[1] T indicates tryptophan; N, niacin.
[2] Mean ± standard deviation.

TABLE 2

Body weight and nitrogen intake, retention, and absorption of cats fed corn or lime-treated corn diets

Experiment no.	Treatment[1]	Average body weight		Av. nitrogen intake	Av. nitrogen retention	Av. nitrogen absorption
		Initial	Final			
		gm	*gm*	*gm*	*%*	*%*
1	Corn + T + N	862	943	5.6	28	86
	Lime-treated corn + T + N	860	947	6.2	31	86
2	Corn + N	1105	1123	6.0	16	86
	Lime-treated corn + N	1123	1143	6.0	16	85
3	Corn + T	1192	1246	6.6	32	88
	Lime-treated corn + T	1208	1263	6.6	28	86

[1] T indicates tryptophan; N, niacin.

affected by the different treatments. Weight gains during the balance periods were higher when tryptophan was included in the diet.

DISCUSSION

The urinary N-methyl nicotinamide excretion values indicate that at approximately the same niacin intake, the presence or absence of supplemented tryptophan in the diet had no effect on the excretion of this niacin metabolite in the urine of cats. The niacin contributed by raw corn or lime-treated corn to the diets was 1 mg, namely, 20 and 17% of the total daily niacin intake, for experiments 1 and 2, respectively. If niacin is less available in raw corn than in that lime-treated because it is "bound," the N-methyl nicotinamide excretion of animals fed raw corn should be lower than in those receiving lime-treated corn in which the niacin has been supposedly "freed" by the treatment. The opposite trend was encountered, however, although the differences in N-methyl nicotinamide excretion between the groups receiving raw and lime-treated corn were not significant (table 1). In addition, when niacin was withdrawn from the diet (experiment 3) and all of the niacin was supplied only by raw corn or lime-treated corn, there was no difference between the N-methyl nicotinamide excretion values of groups fed the two diets. These results suggest that the niacin from raw corn and lime-treated corn is utilized to the same extent by the cat, and they also give further proof of the inability of the cat to convert tryptophan into niacin (Carvalho da Silva et al., '52). The nitrogen balance data indicate that tryptophan, under these experimental conditions, maintained nitrogen balance and that niacin had no effect on that metabolic function.

Diets similar to the one used in this study, have been used by other workers (Harper et al., '57–'58) for the purpose of studying amino acid imbalance in experimental animals able to convert tryptophan into niacin. This imbalance could be reversed in certain cases by adding tryptophan or niacin because of the sparing action of the vitamin on the amino acid. The cat could then be used as an experimental animal to study those amino acid imbalances where tryptophan plays an important role.

SUMMARY

The availability of niacin in corn and in lime-treated corn and the utilization of tryptophan as a source of niacin have been studied in cats. Urinary N-methyl nicotinamide and nitrogen balance were measured in cats fed raw corn or lime-treated corn diets supplemented with either tryptophan or niacin or both. Results indicate that, under the experimental conditions, the cat cannot convert tryptophan into niacin and that this species is able to utilize the niacin from corn and lime-treated corn to an equal extent.

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Nutritional Requirements of the Protein-Depleted Chicken.

II. EFFECT OF DIFFERENT PROTEIN DEPLETION REGIMENS ON NUCLEIC ACID AND ENZYME ACTIVITY IN THE LIVER[1]

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It has been demonstrated that animals that have been fasted or that have been subjected to diets varying in protein content will exhibit pronounced changes in liver composition (Addis et al., '36; Ross and Batt, '56; Stanier, '57; Summers and Fisher, '60). Alteration in the activity of various enzyme systems has been found to be associated with liver composition changes by a number of workers (Williams and Elvehjem, '50; Dioguardi and Secchi, '60). Results indicate that, upon protein depletion, the level of some liver enzymes is increased and that of others is decreased; still others show little or no change (Wainio et al., '59).

Rehabilitation of depleted animals may also produce livers of abnormal composition, especially during the initial stages of repletion (Summers and Fisher, '60; Soberson and Sanchez, '61), and may cause changes in certain enzyme systems (Ju and Nasset, '59; Soberson and Sanchez, '61).

Studies on the changes in nucleic acid content of liver tissue during depletion and repletion have been carried out by several workers (Cooper, '53; Campbell and Kosterlitz, '52; Mendes and Waterlow, '58; Williams, '61; Williamson and Guschlbauer, '61). From their reports deoxyribonucleic acid (DNA) concentration per liver cell apparently remains relatively constant regardless of depletion or repletion. Ribonucleic acid (RNA) per liver cell, however, has been shown to decrease substantially on depletion, a change that is well correlated with nitrogen loss and increase in ribonuclease activity (Zigman and Allison, '59). On repletion, an initial increase in RNA synthesis has been reported to take place (Mendes and Waterlow, '58), whereas DNA synthesis does not increase to any appreciable extent until after a lag period of several days.

The present study was initiated to investigate the enzyme and nucleic acid concentrations in the liver of growing chickens depleted of approximately one-third of their body weight by three different depletion regimens. Since previous work from this laboratory (Summers and Fisher, '60) had shown that animals on various depletion regimens exhibited marked differences in body and liver composition, both during depletion and repletion, it was of further interest to see whether such composition changes would be reflected in certain enzyme activities.

EXPERIMENTAL

Male chicks of a Columbian ♀ × New Hampshire ♂ cross were used throughout the study. The birds were fed a complete starting ration from day of hatch until placed on the respective treatment regimens. One group of birds was fed the control ration throughout the experiment and was sampled when body weights of 160, 190, 225 and 325 gm were attained. Animals that were to be placed on the three depletion regimens, namely, an essentially nitrogen-free diet (hereafter referred to as N-free), a diet containing 15% of protein supplied by gelatin only, and complete starvation, were fed the control ration until an average weight of 225 gm was reached.

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At this time they started to receive their respective treatments. The composition of the experimental rations was the same as for the first study of this series (Summers and Fisher, '60).

During depletion, a sample of animals was taken at 190 and 160 gm. When an average weight of 160 gm was reached, all three treatment groups were refed the control diet; and when weights of 225 and 325 gm were attained, they were sampled again. For the time intervals associated with the body weight changes the reader is referred to table 2 in the first article of this series (Summers and Fisher, '60).

When the chicks had reached the desired average weights, they were starved for 4 hours and then weighed; 5 birds per treatment (including the control) that differed by no more than 5 gm in weight from one another were then killed with chloroform, the livers removed immediately, cleared of any adhering tissue, blotted dry, weighed and refrigerated.

Approximately one-half of each liver was retained for enzyme and nucleic acid determination, and the remaining half was used for moisture, fat and nitrogen (N) analyses. Moisture was determined by drying the liver sample to a constant weight in a forced draft oven at 85°C, and the moisture percentage calculated as the difference between wet and dry weights. The dried livers were ground with mortar and pestle, and samples weighed out for fat and N analysis. Fat was determined according to the method outlined by Summers and Fisher ('60). Nitrogen was analyzed by means of a semimicro-Kjeldahl technique. All determinations were run in duplicate on individual livers.

The portion of liver used for enzyme and nucleic acid determinations was homogenized in a glass grinder within an hour after the livers had been removed using 0.9% sodium chloride solution to make approximately a 15% liver homogenate.

Glutamic pyruvic transaminase activity was determined according to the procedure outlined in a technical bulletin.[2] One milliliter of the 15% liver homogenate solution was diluted to 6 ml with 0.9% sodium chloride solution; 0.2 ml of this mixture was then used for the assay.

Cholinesterase activity was determined by modifying the method of Kuroda et al. ('59). One milliliter of the liver homogenate was pipetted into a test tube, followed by 1 ml of a 0.067 M phosphate buffer (pH 7.4). The mixture was allowed to stand at 39°C for 5 minutes and then 0.5 ml of a 0.1 M acetylcholine solution was added, and the resulting mixture incubated at 39°C for 60 minutes. At this time 2 ml of a mixture of equal parts of 2 M hydroxylamine·HCl and 3.5 N NaOH were added. (In the control tubes the liver homogenate was added at this point.) Two milliliters of a 30% trichloroacetic acid solution were then added to each tube, and the mixture centrifuged. One milliliter of the supernatant was next pipetted into 10 ml of a 0.037 M $FeCl_3$ solution in 0.1 N HCl and the quantity of the remaining acetylcholine determined by measuring the ferric-acethydroxamic acid complex at 530 mμ.

Acid and alkaline phosphatase were determined according to the procedure outlined in a technical bulletin.[3] For the determination of alkaline phosphatase activity, 2 ml of the original liver homogenate were diluted to 25 ml and 0.1 ml of this solution taken for assay purposes. Similarly for acid phosphatase, 1 ml of the liver homogenate was diluted to 100 ml and 0.1 ml of this solution taken for the assay.

RNA and DNA were determined according to the method of Mendes and Waterlow ('58).

No attempt was made to obtain absolute values for the cholinesterase activity or for the concentration of RNA and DNA. Relative values for these measurements are expressed as units (optical density × dilution factor) per gram of tissue.

All determinations were conducted in duplicate on individual livers, and the values presented are averages for 5 birds.

In studying the effect of malnutrition on enzyme changes in tissue, the first problem is to establish a standard of reference. Much of the work reported to date has been expressed as enzyme activity per gram of tissue, but results expressed in this unit are often misleading, because they may be in-

[2] Technical Bulletin 505, Sigma Chemical Company, St. Louis, Missouri.

[3] Technical Bulletin 104, Sigma Chemical Company, St. Louis, Missouri.

fluenced by changes in some of the major tissue components rather than in alteration of the enzyme activity under study. For example, in kwashiorkor where a fatty liver condition is very common, the net result would be an increase in the amount of fat per gram of tissue with a decrease of the other liver components. Thus, even if total enzyme activity per liver remained constant, a decrease would be observed when expressed per gram of tissue. In cases of starvation, when fat is being depleted, the reverse situation may occur.

To simplify the problem of interpretation, a number of workers have expressed enzyme activity on a nitrogen or protein basis. Although such a standard of reference will reflect changes in enzyme levels in comparison with other proteins in the tissue, an increase or decrease in tissue N may still suggest enzyme changes that did not actually occur.

Mendes and Waterlow ('58) concluded from their work and that of others (Thomson et al., '53) that because the DNA content of a liver cell remains relatively constant during growth or depletion of an animal, DNA could serve as a good standard of reference for comparing changes in the measured constituent per tissue cell.

It was therefore decided, for this study, to *limit the interpretation of results to the values expressed per unit of DNA.* For a more complete picture, however, in the graphs enzyme changes and RNA concentration are also expressed as per gram of liver and per milligram of N.

Since the moisture, fat, and N values in the present study are similar to those reported by Summers and Fisher ('60) from experiments with animals of similar weights and treated in an identical manner, the absolute values are not presented. These constituents are, however, presented as per unit of DNA, thus accentuating the relative changes that take place on the various treatments.

RESULTS AND DISCUSSION

Liver composition. The total DNA content per liver and the liver fat and nitrogen content expressed per unit of DNA for the three treatment regimens and for the control animals are shown in figure 1. Total liver DNA remained fairly constant during depletion for all three groups, which is in agreement with the results obtained by others in the adult rat (Campbell and Kosterlitz, '52; Thomson et al., '53). On refeeding there appeared to be an initial lag period in the synthesis of DNA with the starved and N-free groups (compare levels at body weights of 225 and 325 gm, fig. 1). This is in agreement with the work of Williamson and Guschlbauer ('61) on the regeneration of wound tissue in rats, showing that DNA does not rise until about 8 days following treatment. Mendes and Waterlow ('58) also observed only a small increase in DNA until after the 6th day of feeding rats a normal diet after being maintained with a low, poor quality protein diet. In the same study, Mendes and Waterlow demonstrated that the DNA concentration remains constant per liver cell during depletion or repletion, so that apparently no new liver cells are formed during the lag period.

The gelatin-fed group showed an immediate rise in total liver DNA following repletion. Whether this would be true of other poor quality proteins fed at similar levels is not known. The DNA increase of all three treatment groups to levels significantly higher than that of control animals of the same weight is probably only a reflection of the greater age of the treated animals.

On depletion, the gelatin-fed and starved groups showed a large, initial loss in liver fat (fig. 1). By contrast, as previously shown (Summers and Fisher, '60), there was a marked increase in liver fat with the N-free regimen. On refeeding, an increase in liver fat content is observed, especially with the starved group. The return to levels below those observed in the control animals at a body weight of 325 gm reflects the increased DNA content of the liver (as distinct from any changes per liver cell) rather than a change in the proximate composition of the liver.

Per unit of DNA all three groups showed a decrease in N content during depletion (fig. 1), this being most pronounced with the N-free regimen. On refeeding there was no increase in N concentration per unit DNA with the gelatin-fed group. For the N-free and the starved groups, however, there was a marked increase on initial

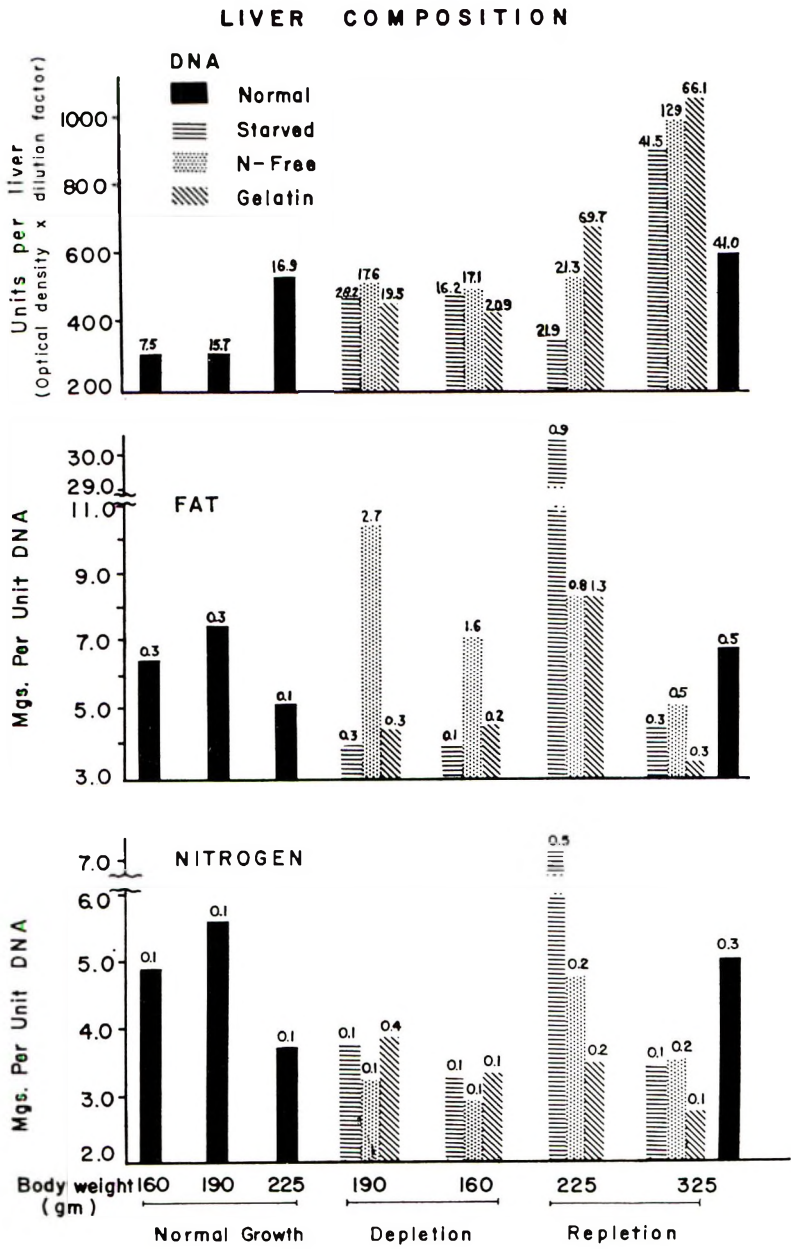

Fig. 1 Deoxyribonucleic acid, fat and nitrogen content of liver from chickens depleted by three methods followed by repletion with a normal ration. Numbers on top of bars represent the standard error of the mean for 5 livers analyzed in duplicate.

repletion followed by a decrease to normal values on continued refeeding. Expressed as percentage of tissue, the N content has been shown to decrease markedly on refeeding of starved animals (Summers and Fisher, '60), but when expressed per unit of DNA, there is apparently a marked increase that parallels that of the fat content.

Liver transaminase. Transaminase activities expressed per gram of liver, per milligram of N, and per unit of DNA are

shown in figure 2. No explanation can be given for the high value noted for the control animals at body weights of 190 gm except to state that the values for all the animals sampled were equally high. During depletion there was a very marked increase in the transaminase activity of the starved group. This agrees with observations on rats (Akehi, '57; Soberson and Sanchez, '61). Akehi postulated that such an increase may be due to an increase in free amino acids resulting from rapid de-

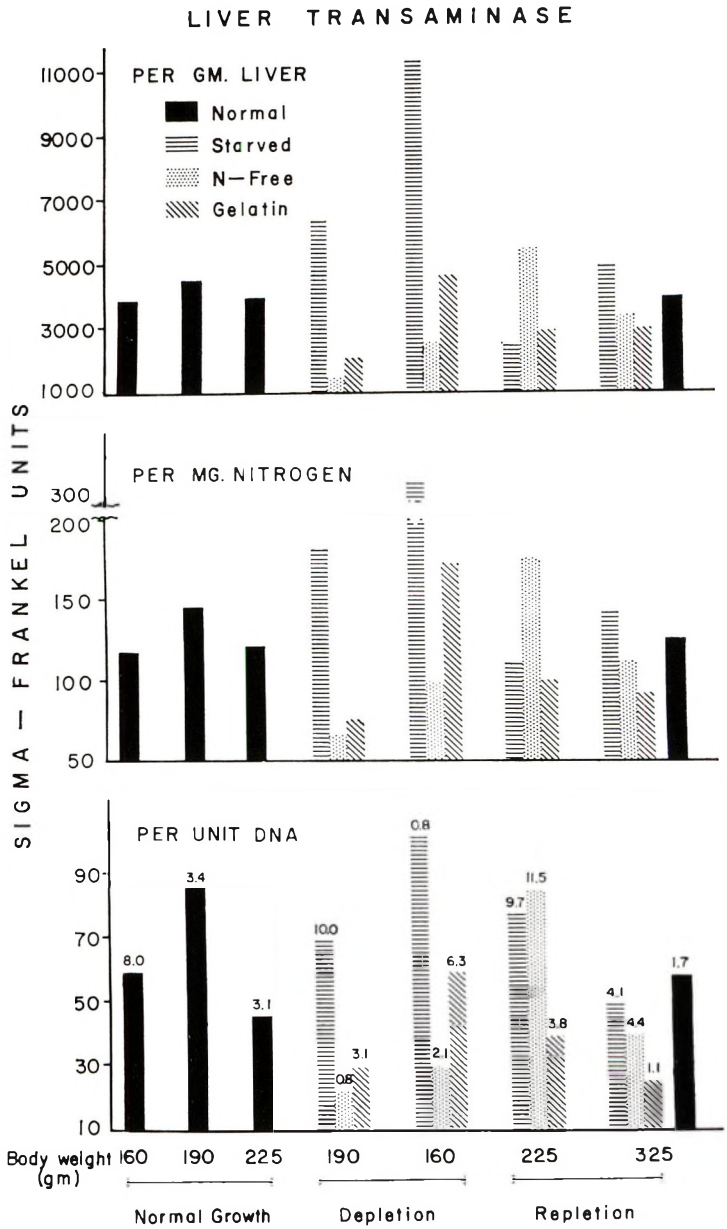

Fig. 2 Liver transaminase activity from chickens depleted by three methods followed by repletion with a normal ration. Numbers on top of bars represent the standard error of the mean for 5 livers analyzed in duplicate.

struction of tissue protein during starvation. The decreased activity on refeeding the starved animals could then be explained by an arrest of tissue breakdown.

With the N-free and gelatin-fed groups, depletion caused an initial drop in activity. Since these animals received a liberal amount of dietary energy, anticipated tissue destruction would be very low (total depletion time was two weeks for the N-free and gelatin-fed groups versus 6 days for the starved group). As depletion pro-

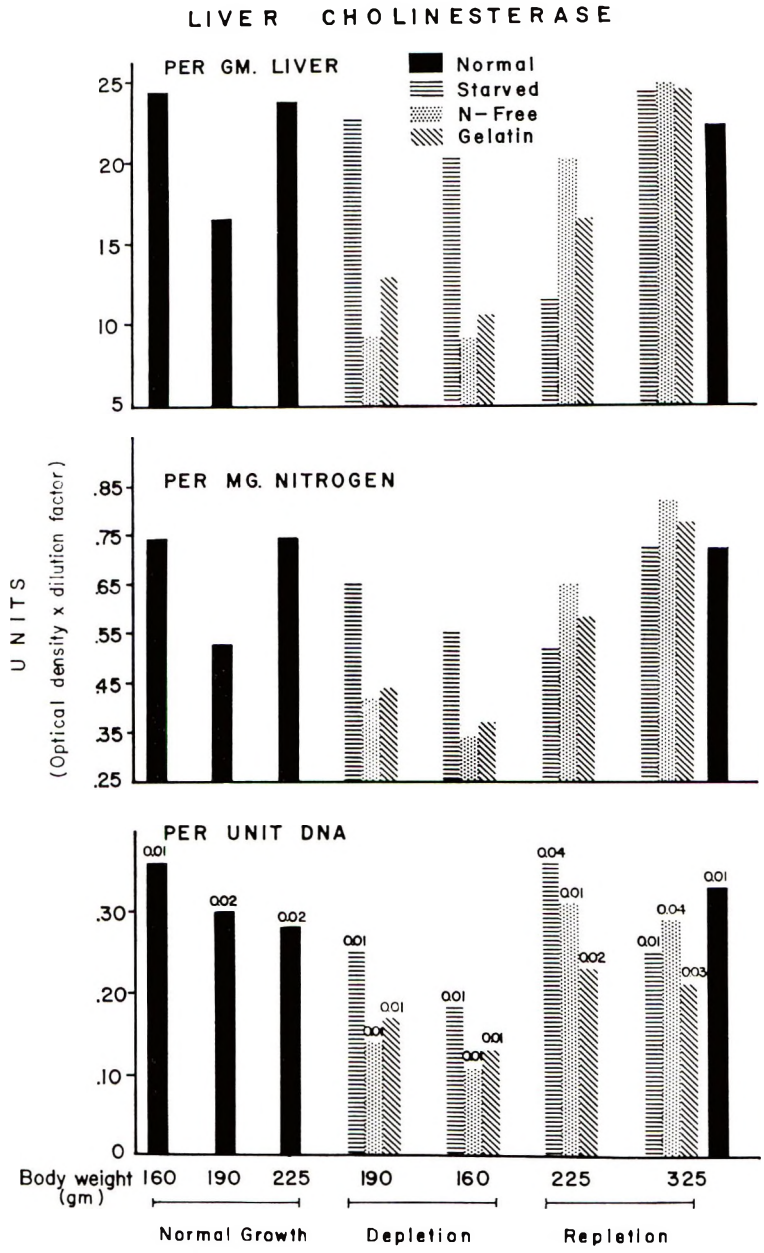

Fig. 3 Liver cholinesterase activity from chickens depleted by three methods followed by repletion with a normal ration. Numbers on top of bars represent the standard error of the mean for 5 livers analyzed in duplicate.

gressed, an increase in transaminase activity, particularly for the gelatin-fed group, was noted. This may be a reflection of a reduced food intake resulting in increased tissue protein breakdown and for the gelatin-fed group the catabolism of the absorbed but unusable amino acids as well.

On initial refeeding, the N-free group exhibited a substantial increase in activity while that of the other two groups de-

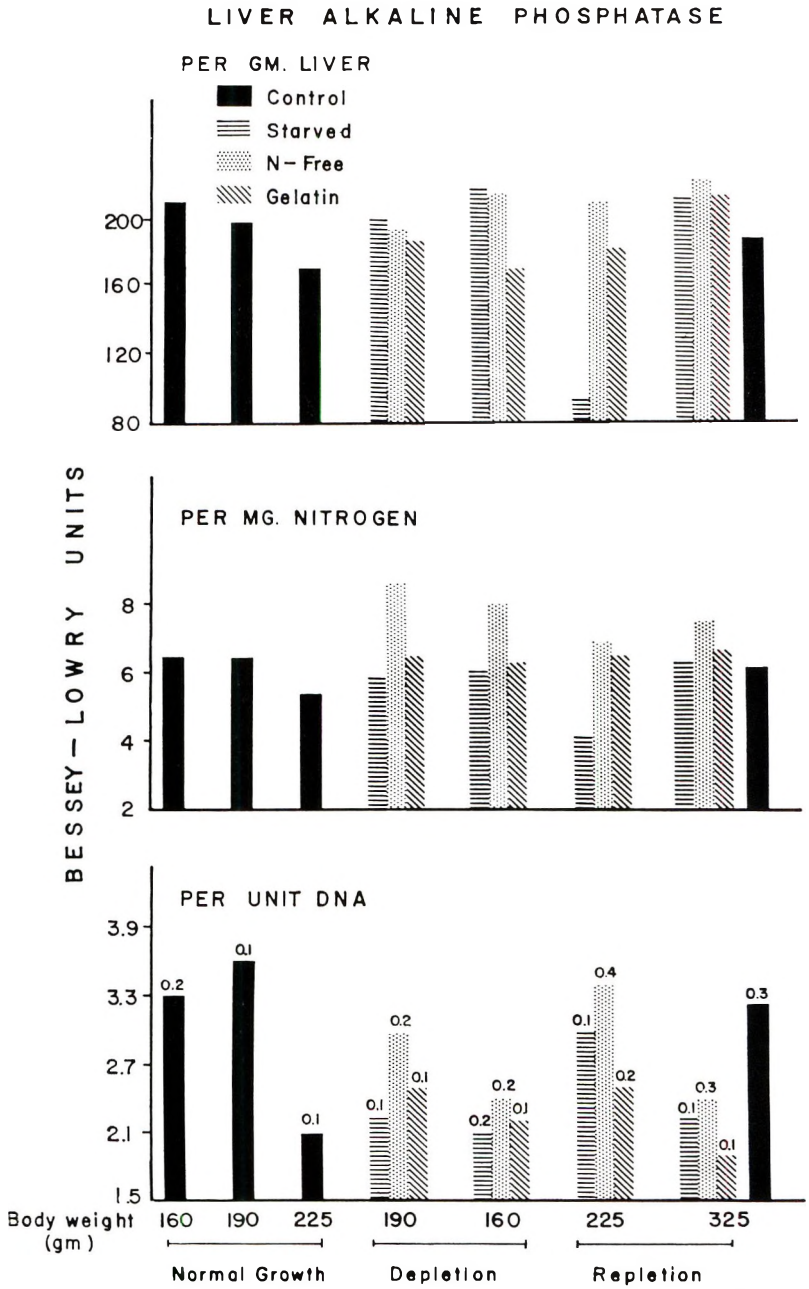

Fig. 4 Liver alkaline phosphatase activity from chickens depleted by three methods followed by repletion with a normal ration. Numbers on top of bars represent the standard error of the mean for 5 livers analyzed in duplicate.

creased. This increase may be explained by the sudden availability of dietary N, which the animals were rapidly converting into tissue protein. Such an explanation is strengthened by the observations of Akehi ('57) that liver transaminase of rats is increased by feeding a high protein diet as compared with a low protein one. With the gelatin-fed and starved groups, refeeding resulted in a decrease in transaminase activity. Although no obvious explanation can be offered, these observations are most likely related to the differences in fat-to-nitrogen ratios at the end of depletion of the gelatin and starved groups versus the N-free group (Summers and Fisher, '60).

Liver cholinesterase. The relative values for cholinesterase activity are shown in figure 3. During depletion, a progressive reduction in activity was noted with all three treatment groups, followed by a sharp increase on repletion. Similar changes were observed in the livers of children suffering from kwashiorkor when biopsied on admission to the hospital and again after treatment with an adequate diet (Waterlow, '50; Mukherjee and Sarkar, '58).

Liver alkaline phosphatase. Results for the liver alkaline phosphatase activities are shown in figure 4. All three treatment groups exhibit a similar pattern. Compared with the controls at 225 gm of body weight, there was an initial increase on depletion, followed by a small decrease. A similar pattern was noted on repletion. These changes in the growing chick follow those observed in rats that had been starved and then refed normal diets (Soberson and Sanchez, '61), and also parallel those noted in the serum of kwashiorkor-stricken children.[4] The decrease to activity levels substantially below those of the control animals at 325 gm of body weight can be explained by the higher level of DNA in the livers of the repleted birds. In general, the variation in enzyme activity (including that of the controls, during the initial period of normal growth) is such as to preclude interpretations. Also, changes in activity were noted when expressed as per gram of liver or per milligram N. As we pointed out earlier, different and perhaps misleading interpretations could easily be made if the experimental values are not related to a constant tissue component such as DNA.

Acid phosphatase. Acid phosphatase values are presented in figure 5. The starved and gelatin-fed group activities behaved similarly during depletion, showing a continual decrease. The N-free animals, however, showed an increase in activity with initial depletion which was followed by a decrease to a value in line with that of the other groups. On repletion there was no change in the gelatin-fed or N-free animals, whereas the starved chicks exhibited an initial increase, followed by a decline to the activity levels of the other treatment groups. The similarity between the activity pattern for the starved and gelatin-fed groups during depletion follows closely the parallel liver composition pattern of these animals in contrast with that of the N-free group. Also, the liver composition pattern of the gelatin-fed and N-free groups, on repletion, are similar to each other, in contrast with that of the starved group (Summers and Fisher, '60).

Liver RNA. The relative concentrations of RNA as expressed by the three bases of reference are shown in figure 6. During the depletion period there was a decrease in RNA per unit DNA for all three treatment groups. Similar changes have been reported in the rat (Davidson and Leslie, '45; Cooper, '53; Mendes and Waterlow, '58). On refeeding, all groups exhibited an initial increase in RNA, the increase being more pronounced in the starved group. On continued refeeding the RNA concentration of all groups decreased to similar values. An increase in the RNA/DNA ratio has been reported during repletion (Mendes and Waterlow, '58; Williamson and Guschlbauer, '61). Specifically, these workers observed that RNA increased rapidly, whereas a lag period of several days occurred before any noticeable rise in DNA concentration was noted.

GENERAL COMMENTS

The present study indicates that the standard of reference used in expressing enzyme activity may have a marked influence on the interpretation of results.

[4] Scrimshaw, N. S., M. Beher, M. Guzmán, F. Viteri and G. Arroyave 1955 Biochemical and hematological findings in infantile pluricarencial syndrome (kwashiorkor). Federation Proc., *14*: 449 (abstract).

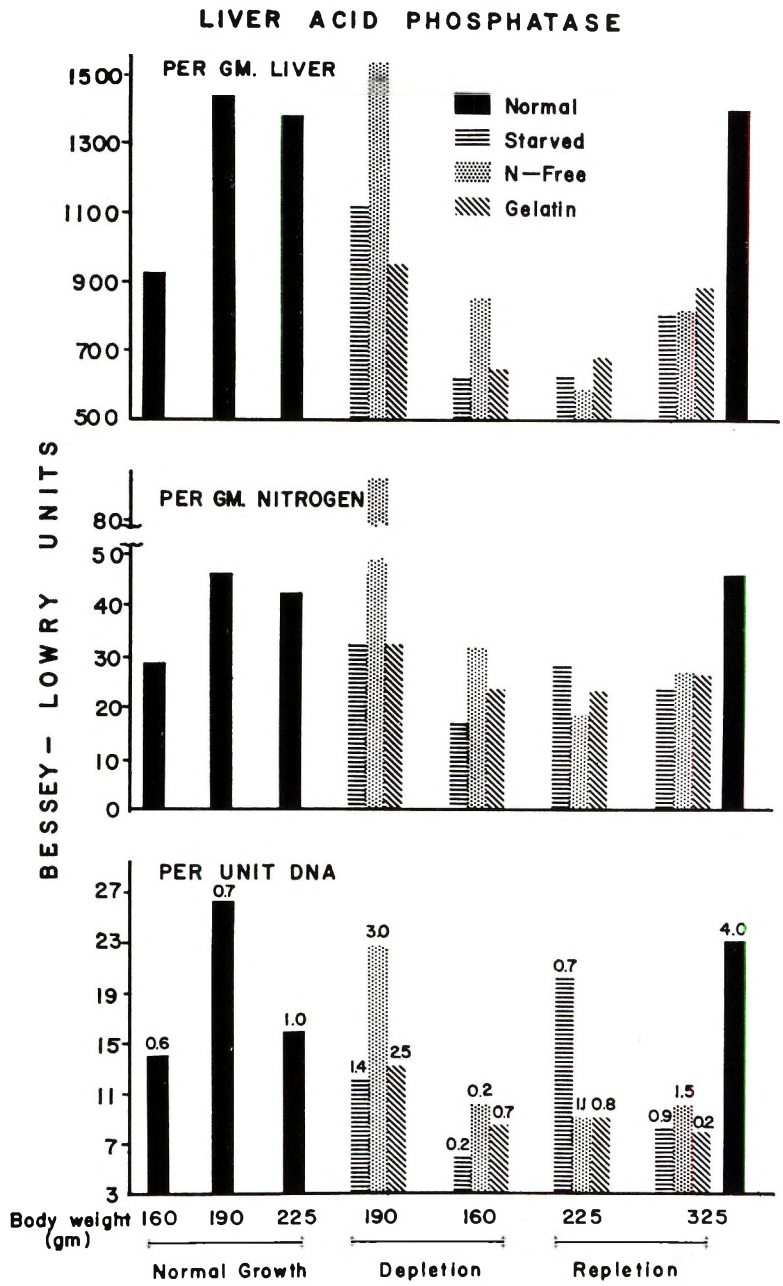

Fig. 5 Liver acid phosphatase activity from chickens depleted by three methods followed by repletion with a normal ration. Numbers on top of bars represent the standard error of the mean for 5 livers analyzed in duplicate.

Thus, little change occurred in alkaline phosphatase activity when expressed as per gram of liver or per milligram of N, whereas marked changes occurred when the activity was expressed as per unit of DNA.

The results indicate the possible usefulness of distinguishing certain nutritional

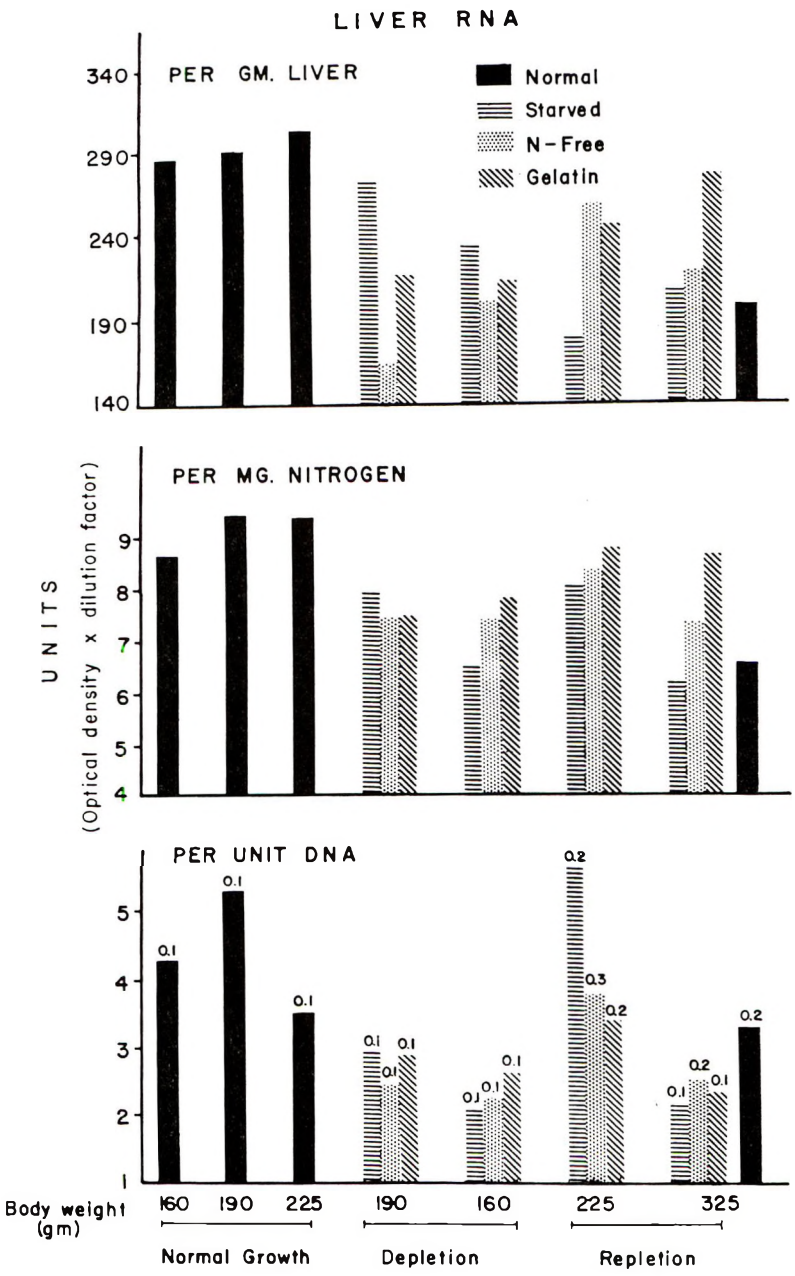

Fig. 6 Liver ribonucleic acid content from chickens depleted by three methods followed by repletion with a normal ration. Numbers on top of bars represent the standard error of the mean for 5 livers analyzed in duplicate.

states through the study of certain liver enzyme activities. Transaminase activity, in particular, seems very sensitive in this regard.

Acid phosphatase levels also varied with the type of depletion. On initial depletion the N-free group exhibited an increase in activity, whereas the other two groups

showed a decrease. On refeeding, however, the starved animals showed an initial increase in enzyme level, whereas that of the N-free and gelatin-fed groups remained unchanged.

It is interesting to speculate on the possibility of using changes in enzyme concentration to determine the extent and the type of depletion in malnourished subjects. In the present work, the starved animals exhibited changes in body composition similar to those reported in children suffering from marasmus. During the early stages of malnutrition, an increase in transaminase activity might well indicate that a subject is suffering from marasmus, whereas a decrease in activity would point to the possibility of kwashiorkor, since composition changes during this condition resemble those of the animals depleted with a N-free diet.

Most of the enzyme activities studied exhibited some degree of concentration reversal during the two stages of repletion that were examined. It may therefore be helpful to follow the course of certain enzymatic activities during a repletion treatment as a check whether protein synthesis is progressing normally.

SUMMARY

Three groups of growing chickens were depleted to two-thirds of their initial body weight (225 gm) by subjecting them to (a) complete starvation, (b) a N-free diet and (c) a diet supplying 15% of protein as gelatin; after depletion, birds were sampled at 190 and 160 gm; on repletion, animals were sampled at 225 and 325 gm. A group of control birds was sampled at identical weights.

Livers were analyzed for moisture, fat, nitrogen, transaminase, cholinesterase, alkaline and acid phosphatase, RNA and DNA. Results were expressed as per unit of liver, nitrogen and DNA, but discussed primarily as per unit of DNA. During depletion and repletion, changes were noted in liver composition (fat, N and moisture) among treatment groups. Enzyme and nucleic acid concentrations also differed with the type of depletion not only during the depletion period but also on refeeding. Liver transaminase and RNA were particularly affected and sensitive to the depletion regimens.

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Effects of B Vitamins on Protein Utilization from Rice-Legume Dietaries by the Growing Rat[1]

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Several reports point out the beneficial effects of higher dietary supplements of B vitamins on the efficiency of protein utilization. The literature on the subject has been extensively reviewed in recent communications from this laboratory (Marfatia and Sreenivasan, '60a, b; Fatterpaker et al., '60; Wagle and Sreenivasan, '61). These studies have shown that B vitamins at optimal levels improve utilization of protein in growing rats fed diets containing single and mixed proteins. These observations have been extended to demonstrate the effects due to vitamin B_{12} and folic acid, and the impairment in growth and nitrogen retention in rats arising from split-feeding of protein and carbohydrate components of the diet, as well as from intermittent feeding of high and low protein diets. These could be offset by using an optimal supplement of B vitamins in the diet. Studies with protein-starved rats have demonstrated that the turn-over of liver and plasma constituents is influenced by the levels of certain B vitamins, more specifically of folic acid and vitamin B_{12} (Wagle and Sreenivasan, '60).

Our knowledge of the optimal requirements for essential amino acids for laboratory animals and man and of the amino acid content of dietary constituents makes it possible to supplement diets with amino acids deficient in them (Sure, '53, '57a; Westerman et al., '57). Likewise, small supplements of a high quality animal protein have been used frequently in diets to make up the nutritional deficiencies in low quality proteins (Sure, '57b, c; Westerman et al., '57; Deshpande et al., '55).

The object of the present investigation was to assess the effects of B vitamin, amino acid and high quality protein supplementation of a poor quality diet typical of that consumed by low income groups of South India. This diet, conforming to the average local practice, included rice and a legume (*Cajanus indicus*) as major constituents with leafy and nonleafy vegetables, milk and also condiments in quantities as normally used. The cereal:legume proportions were blended to give diets containing two protein levels.

EXPERIMENTAL AND RESULTS

In all experiments, Wistar rats of either sex were housed individually in raised mesh-bottom cages, with access to the diets in excess quantities and in scatter-proof cups.

Two isocaloric diets containing 6.8% (diet 1) and 10.2% (diet 2) of total protein as determined by analyses for total nitrogen by the Kjeldahl method were prepared. The composition of these diets is presented in table 1. Rice and legume (*Cajanus indicus*) contributed 5.22 and 1.15% respectively, of protein to diet 1 and 2.62 and 6.90% protein to diet 2, the rest of the protein being derived from the other constituents of the diet. The vegetables were macerated and blended with the other ingredients. The diets were steamed for one-half hour in an autoclave and later dried under vacuum at 70°C for 5 hours. The dried material was pulverized and stored in the cold for use.

The B vitamins, where provided, were added at one of two levels (table 1), which corresponded to the optimal (high) and minimal (low) requirements of these vitamins for the growing rat (Marfatia and Sreenivasan, '60a).

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TABLE 1

Composition of the rice-legume diets[1]

Components	Diet 1	Diet 2
	gm	*gm*
Milled white rice	74.0	36.5
Legume (*Cajanus indicus*)	5.0	30.0
Fresh milk	5.0	10.0
Nonleafy vegetables[2]	8.0	10.0
Leafy vegetables[3]	2.0	5.0
Spice mixture[4]	2.5	2.5
Arachis oil[5]	2.5	5.0
Sucrose or vitaminized sucrose	1.0	1.0

Vitaminized sucrose (B vitamins per gm of sucrose)

	High level	Low level
	mg	*mg*
Thiamine·HCl	0.3	0.015
Riboflavin	0.4	0.015
Pyridoxine·HCl	0.3	0.015
Ca pantothenate	1.0	0.1
Niacin	2.0	0.1
Biotin	0.05	—
Folic acid	0.1	0.003
Vitamin B_{12}	0.015	—
Inositol	20.0	5.0
Choline Cl	20.0	5.0

[1] Where vitaminized sucrose was added, addition of B vitamins was made either at high or low level as indicated.
[2] Consisting of edible portions of eggplant (*solanum melogena*) and okra (*Hibiscus esculentus*) in equal proportion.
[3] Consisting of amaranth (*Amaranthus gangeticus*) and spinach (*spinacia oleracea*) in equal proportion.
[4] Consisting of (parts): sodium chloride, 1; tamarind fruit pulp, 0.7; pepper, 0.1; chillies, 0.1; turmeric, 0.1; and cinnamon, 0.1; with additions of calcium (as calcium lactate) and iron (as ferrous ammonium sulphate) at levels of 50 and 2 mg, respectively, per 100 gm of the spice mixture.
[5] Fat-soluble vitamins: α-tocopherol, menadione, vitamin A acetate and vitamin D (calciferol) were added to the diet in amounts (per 100 gm diet) of 5, 1, 0.31 and 0.0045 mg, respectively.

The diets were analyzed for their essential amino acid content. One-gram samples were hydrolyzed in 25-ml sealed glass bulbs with 10 ml of 6 N HCl for 10 hours at 15 p.s.i. The hydrolysates on cooling were filtered and adjusted to pH 7.0.

The essential amino acids, other than tryptophan, were assayed microbiologically according to Barton-Wright ('52). Tryptophan was determined in enzymic digest of the diets. Samples equivalent to one gram of protein were suspended in 50 ml of water and, after adjustment of the pH to 1.5 with 6 N HCl, the suspensions were hydrolyzed with 10 mg of pepsin[3] at 37° for 18 hours. Hydrolysis was continued with 10 mg of trypsin[4] at 40° for 24 hours after addition of 3 gm of KH_2PO_4 and adjustment of pH to 8.4 The hydrolysate was finally neutralized and washed with ether to remove indole, anthranilic acid, etc., made to volume and filtered. Assay of tryptophan in the filtrate was according to Barton-Wright ('52). The results are tabulated with the amino acid requirement for normal growth in the rat (Rose, '37) (table 2). Diet 1 is deficient in all the essential amino acids except arginine and valine, whereas diet 2 is limiting in only three amino acids — methionine, lysine and tryptophan.

Data on food consumption and body weight were recorded during the 8-week period. The liver analyses for total and nonprotein nitrogen and total lipids, as well as determinations of total and nonprotein nitrogen in blood plasma, were carried out at the end of each experimental period according to methods detailed elsewhere (Marfatia and Sreenivasan, '60a). The protein-free filtrates of plasma were used in one experiment for the assay of free essential amino acids; the filtrates were treated for this purpose in the same manner as were the diet hydrolysates.

TABLE 2

Essential amino acid composition of the rice-legume diets[1]

Amino acid[2]	Diet 1	Diet 2	Requirement for normal growth (Rose, '37)
	Percentages of diet		
Arginine	0.44	0.8	0.2
Histidine	0.19	0.38	0.4
Isoleucine	0.38	0.66	0.5
Leucine	0.52	0.78	0.8
Lysine	0.35	0.8	1.0
Methionine	0.3	0.4	0.6
Phenylalanine	0.48	0.96	0.7
Threonine	0.3	0.64	0.5
Tryptophan	0.08	0.08	0.2
Valine	0.9	1.3	0.7

[1] *Leuconostoc mesenteroids* P 60 was used for the assay of lysine, histidine, methionine and phenylalanine; *Lactobacillus arabinosus* 17-5 was used for the assay of tryptohan, leucine, isoleucine and valine; arginine and threonine were assayed using *Streptococcus faecalis*.
[2] L-forms of lysine, histidine and arginine and DL-forms of methionine, tryptophan, threonine, phenylalanine, leucine, isoleucine and valine were used as standards. Values expressed are, however, in terms of the L-amino acid (50% in the case of racemic standards).

[3] Nutritional Biochemicals Corporation, Cleveland.
[4] See footnote 3.

Supplementation with B vitamins. Weanling male rats weighing 40 to 45 gm were used in a pilot experiment. The animals were divided into 4 groups of 6 rats each and fed diets 1 and 2 supplemented with B vitamins at the high level or unsupplemented. Although significant increases in the growth rate and in the efficiency of protein utilization were obtained by increasing the level of dietary protein, or, at the high protein level, by increasing the B-vitamin concentration, the diets failed to support good growth in general (table 3). This effect could be attributed to the poor over-all quality of the protein in these diets; and since the amino acid requirements of immature animals for optimal growth are more exacting than those of young adult ones for normal growth (Hegsted and Worcester, '47), the above experiment was repeated using male rats of 90- to 95-gm initial weight 6 to a group. Data were also obtained in this experiment on total and nonprotein nitrogen in the liver and nonprotein nitrogen and free essential amino acids in blood plasma. Results on protein efficiency ratio and liver constituents are included in table 3. A marked over-all improvement in growth may be seen from this table. Increased protein concentration and vitamin supplementation considerably enhanced both growth and protein efficiency ratio. There was also a striking increase in the liver total nitrogen concentration as a result of increasing the dietary protein level; vitamin supplementation produced relatively small gains. The liver nonprotein nitrogen concentration was not influenced by the dietary protein level but was reduced as the diet was fortified with B vitamins. There was a decrease in nonprotein nitrogen in the rats fed the high vitamin diets, the effect being more at the high protein level (table 4). These changes were associated with significant reductions in plasma levels of histidine, methionine and threonine. It may be noted that, as a result of increasing the dietary protein, the concentration of every amino acid in the plasma was also increased when the diets were not supplemented with B vitamins; in the presence of B vitamins, reductions in methionine and threonine concentrations were observed despite raising the protein level.

TABLE 3

Effect of diet and B-vitamin supplementation on growth and protein efficiency in weanling and young adult rats

Group		Weanling rats			Young adult rats				
								Liver[4]	
Protein	B vitamin[1]	Gain in weight[2]	Food intake	PER[3]	Gain in weight	Food intake	PER	Total nitrogen	Non-protein nitrogen
%		*gm*	*gm/day*		*gm*	*gm/day*		*mg/gm*	*mg/gm*
6.8	−	11.0 ± 5.5[5]	3.8 ± 0.2	0.77 ± 0.04	18.2 ± 3.1	5.8 ± 0.2	0.82 ± 0.04	21.3 ± 0.7	2.5 ± 0.02
6.8	+	16.7 ± 4.9	4.1 ± 0.3	1.07 ± 0.02	28.3 ± 3.6	6.7 ± 0.4	1.11 ± 0.05	25.1 ± 0.8	2.3 ± 0.05
10.2	−	23.8 ± 3.4	3.9 ± 0.2	1.06 ± 0.05	34.1 ± 2.6	5.5 ± 0.3	1.08 ± 0.02	29.4 ± 0.7	2.4 ± 0.03
10.2	+	36.8 ± 3.4	4.3 ± 0.3	1.50 ± 0.07	50.2 ± 5.9	6.4 ± 0.5	1.37 ± 0.04	32.7 ± 0.6	2.2 ± 0.04

[1] The diets were with (+) or without (−) a supplement of B vitamins at the high level as indicated in table 1.
[2] Mean weight gains per rat over a period of 8 weeks.
[3] Protein efficiency ratio, expressed as grams gain per gram of protein consumed.
[4] Values are expressed on the fresh weight of tissue.
[5] Average of 6 rats/group ± standard error of the mean.

TABLE 4

Effect of diet and B-vitamin supplementation on plasma nonprotein nitrogen and free amino acids[1]

Group[2]		Non-protein nitrogen	Arginine	Histidine	Iso-leucine	Leucine	Lysine	Methio-nine	Phenyl-alanine	Threonine	Trypto-phan	Valine
Protein	B vitamins[3]											
%		*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
6.8	–	27.0 ± 1.2[4]	2.41 ± 0.06	0.54 ± 0.01	0.58 ± 0.09	1.62 ± 0.13	5.21 ± 0.07	0.40 ± 0.07	1.41 ± 0.15	4.07 ± 0.42	1.18 ± 0.12	2.32 ± 0.25
6.8	+	24.8 ± 0.8	2.32 ± 0.08	0.44 ± 0.04	0.61 ± 0.11	1.56 ± 0.03	5.34 ± 0.91	0.32 ± 0.04	1.43 ± 0.21	3.82 ± 0.38	1.21 ± 0.16	2.46 ± 0.31
10.2	–	27.2 ± 1.4	2.72 ± 0.14	0.74 ± 0.05	0.72 ± 0.06	1.82 ± 0.16	6.28 ± 0.61	0.65 ± 0.02	2.12 ± 0.36	4.59 ± 0.21	1.51 ± 0.18	2.91 ± 0.24
10.2	+	21.2 ± 0.9	2.61 ± 0.21	0.59 ± 0.01	0.68 ± 0.08	1.61 ± 0.19	6.04 ± 0.58	0.21 ± 0.04	2.21 ± 0.36	2.82 ± 0.21	1.48 ± 0.16	3.04 ± 0.23

[1] For standards, L- forms of lysine, histidine and arginine and DL-forms of methionine, tryptophan, threonine, phenylalanine, leucine, isoleucine and valine were used. Values expressed are, however, in terms of the L- amino acids (50% of the racemic standards).

[2] Each group consisted of 6 young adult rats.

[3] Diets were supplemented with (+) or without (–) the B vitamins at the high level as given in table 1.

[4] Mean value ± standard error of mean.

Diet 1 was eliminated from subsequent experiments owing to its general poor quality, diet 2 serving as the basal ration in these studies.

Amino acid supplementation and B vitamins. Female rats weighing 90 to 95 gm were divided into 6 groups of 6 rats each. One group was fed the basal 10.2% protein ration (diet 2) without additional supplementation of B vitamins. Another group subsisting on this ration received supplements of L-lysine (0.2% of diet), DL-methionine (0.4%) and DL-tryptophan (0.24%). The remaining 4 groups subsisted on the above rations as supplemented with B vitamins at high or low levels (table 1). Data were obtained on protein efficiency ratio and on liver content of total and nonprotein nitrogen and total lipids (table 5).

The efficiency of protein utilization was improved to a greater extent with amino acid supplementation than with the B-vitamin supplement. The animals fed the amino acid-supplemented diet were further benefited by incorporation of B vitamins in the diet. The gains in liver total nitrogen due to B vitamins alone were again small, whereas with amino acid supplementation, these were appreciable. A further increase in liver nitrogen content was obtained when the diet was supplemented with both amino acids and B vitamins at a high level. The nonprotein nitrogen constitutents were apparently not significantly affected by enriching the diet with amino acids. However, with even a low level of B vitamins added to the enriched diet, there was a marked reduction in liver nonprotein nitrogen. With both high and low level supplements of B vitamins the change in nonprotein nitrogen was of the same order. There was a small but significant decrease in liver lipids when the basal ration was supplemented with B vitamins, the change being of equal magnitude at high- or low-level supplementation. A marked decrease in lipids was brought about by amino acid supplementation and, still further, when additional B vitamins were provided at the high level.

Egg albumen supplementation and B vitamins. In another set of experiments, egg albumen replaced 2% of the total legume proteins (6.9%) in the basal ration. The basal ration (diet 2), and also the modified ration, were supplemented with B vitamins at the two levels, high and low. Four groups of male rats, with 6 rats of approximately 95-gm average weight per group, subsisted on these rations for 8 weeks.

The results indicate a marked increase in the efficiency of protein utilization following egg albumen substitution (table 6). The magnitude of the increase in the protein efficiency ratio obtained by raising the dietary B-vitamin level was, however, somewhat lower in the animals receiving egg albumen than in the control animals. These results are substantiated by data on the liver constituents. The B-vitamin level had no significant influence on these constituents in the modified ration containing egg albumen. On the other hand, there were significant reductions in liver nonprotein nitrogen and liver lipids when the vitamin level in the basal ration was increased; also there was a small but significant increase in liver total nitrogen. With egg albumen there was also a marked decrease in liver lipids and in liver nonprotein nitrogen and improvement in protein nitrogen content of the tissue.

DISCUSSION

The improvements in growth rate ob tained with B-vitamin supplements might indicate that the basal diet was lacking in one or more of them; however, no syndromes of any B-vitamin deficiency were manifest. The adult rat can, it is known, survive in a reasonably healthy state on very low amounts of certain B vitamins (Miller and Baumann, '44). On the other hand, increased intakes of B vitamins have been shown to result in improved growth (Sure and Romans, '48; Sure, '50; Marfatia and Sreenivasan, '60b). The observed increase in protein nitrogen and decrease in nonprotein nitrogen constituents in the liver with a higher intake of B vitamins are again indicative of a more efficient protein utilization. Vitamin supplementation also lowers concentrations of plasma nonprotein nitrogen and certain free amino acids. Similar observations (Charkey et al., '50, '54) with chicks in relation to vitamin B_{12} have been interpreted to suggest a function for the vitamin in anabolic processes, probably involving channeling of amino

TABLE 5

Effect of B-vitamin and amino acid supplementation of rice-legume diet at 10.2% of protein on growth and liver composition[1]

Supplements[2]			Gains in weight	Food intake	Protein efficiency ratio[3]	Liver		
B vitamins (low level)	B vitamins (high level)	Lysine, methionine and tryptophan				Total nitrogen	Non-protein nitrogen	Total lipids
			gm	*gm/day*		*mg/gm fresh weight*		
−	−	−	36.5 ± 4.1[4]	6.0 ± 0.5	1.06 ± 0.05	25.5 ± 0.4	2.6 ± 0.04	129.6 ± 2.7
+	−	−	42.3 ± 4.3	6.2 ± 0.6	1.19 ± 0.01	26.3 ± 0.5	2.4 ± 0.05	122.1 ± 2.9
−	+	−	52.0 ± 3.3	6.5 ± 0.2	1.40 ± 0.02	27.4 ± 0.6	2.3 ± 0.06	123.8 ± 3.1
−	−	+	58.5 ± 5.6	7.1 ± 0.4	1.44 ± 0.004	29.5 ± 0.5	2.5 ± 0.01	99.8 ± 2.6
+	−	+	72.2 ± 3.2	8.3 ± 0.4	1.52 ± 0.03	30.8 ± 0.6	2.2 ± 0.03	102.7 ± 4.2
−	+	+	83.0 ± 5.6	8.0 ± 0.2	1.82 ± 0.05	32.2 ± 0.5	2.1 ± 0.02	95.0 ± 1.8

[1] Data obtained over a period of 8 weeks with 6 young adult rats per group.
[2] Supplements were made (+) or not (−) to the rice-legume diet at 10.2% of protein. The levels of B vitamins were as indicated in table 1. Amino acids, L-lysine, DL-methionine and DL-tryptophan were added at 0.2, 0.4 and 0.24% of diet, respectively.
[3] As defined in table 3.
[4] Mean values and their standard errors.

TABLE 6

Effects of B-vitamin and egg albumen supplementation of the rice-legume diet at 10.2% of protein on growth and liver composition

Supplements[1]	Weight gained in 8 weeks	Food intake	Protein efficiency ratio[2]	Liver		
				Total nitrogen	Non-protein nitrogen	Total lipids
	gm	*gm/day*		*mg/gm fresh weight*		
B vitamins (low level)	45.0 ± 1.2[3]	7.6 ± 0.34	1.04 ± 0.03	28.2 ± 0.52	3.2 ± 0.12	95.3 ± 1.5
B vitamins (high level)	63.6 ± 3.7	7.8 ± 0.49	1.43 ± 0.05	29.3 ± 0.44	2.9 ± 0.21	84.0 ± 1.7
Egg albumen + B vitamins (low level)	72.6 ± 3.5	7.5 ± 0.26	1.66 ± 0.02	29.8 ± 0.74	2.7 ± 0.10	77.6 ± 1.9
Egg albumen + B vitamins (high level)	91.5 ± 5.2	8.2 ± 0.28	1.95 ± 0.08	31.5 ± 0.30	2.8 ± 0.13	76.8 ± 1.7

[1] The basal rice-legume diet at 10.2% of protein was supplemented with B vitamins either at low or high level as indicated in table 1. Egg albumen addition was at 2% of protein at the expense of an equal amount of legume protein.
[2] As defined in table 3.
[3] Mean value of 6 animals/group ± standard error of mean.

acids into tissue protein synthesis. In chicks, an effect of dietary vitamins in lowering certain plasma amino acids, while raising others, has been reported by Richardson et al. ('53). The decrease in plasma concentrations of histidine, threonine and methionine with the vitamin supplementation would imply either that the vitamins have a direct functional significance in the metabolism of these amino acids or that a general imbalance of amino acids, reflected in plasma concentrations, is offset, at least to some degree, by higher intake of the B vitamins; it is possible that both causes may be simultaneously operating.

The improvement in growth resulting from supplementation of the diet with its deficient amino acids occurred as expected. Similar nutritional improvements have been observed by others with rice diets (Pecora and Hundley, '51; Pecora, '53; Harper et al., '55). The observed effects of the B vitamins in the presence of the deficient amino acids could mean that for the B vitamins to function, a certain adequacy of dietary amino acids is essential. Probably one aspect of this function is the better utilization of histidine, methionine and threonine as suggested by decreased plasma levels of these amino acids in the previous experiment.

Accumulation of fat in the liver has been suggested as due to the presence of amino acids in the diet in proportions that cannot be utilized by the animal for protein synthesis, the unutilized amino acids contributing to lipogenesis (Harper et al., '55). Partial deficiencies of lysine and threonine have been characterized by such liver fat accumulation (Harper et al., '53; Singal et al., '53). Hence, correction of amino acid deficiencies in the diet may be expected to result in the observed lowering of liver fat. The B vitamins at both high and low levels seem to have relatively little influence on liver lipids. Sarett and Perlzweig ('43) also observed that B-vitamin supplementation of a low protein diet had little influence on liver lipids.

The increased growth obtained with partial substitution of egg albumen for legume protein in the rice legume diet is obviously the result of an improvement in protein quality. The capacity of a protein to promote growth depends essentially upon the adequacy with which the quantity and proportions of the amino acids made available by digestion and absorption match the quantity and proportions needed for tissue synthesis and repair. Similar responses in growth have been observed by other workers (Harper et al., '55) using rice diets supplemented with small amounts of animal protein. In fact, supplementation with animal protein was much superior to that with essential amino acids, the latter often causing imbalances (Deshpande et al., '55). Kik ('56) has demonstrated that fish flour in small quantities added to a rice diet is beneficial for growth. Carpenter and his associates ('57) also reported that fish flours make effective supplements to cereal diets.

The increased growth obtained with the optimal level of B vitamins would indicate the inadequacy of dietary intakes of vitamins when the diets are supplemented with minimal quantities of vitamins. With addition of egg albumen and consequent improvement in the over-all protein quality, the effects due to vitamins were less pronounced. Similar observations have been reported earlier from this laboratory using wheat flour-gluten and wheat flour-egg albumen diets (Marfatia and Sreenivasan, '60).

SUMMARY

The rice-legume diets typical of those consumed by a large section of low-income groups in India were prepared to contain 6.8 and 10.2% of protein and fed to rats for 8 weeks.

Although the diets failed to support good growth in weanling rats, growth was appreciable with young adult rats, and was further improved by B-vitamin supplementation. There were significant reductions in plasma levels of histidine, methionine and threonine in the B-vitamin supplemented group. The amino acid analysis of the diets indicated that the 6.8% protein diet was deficient in amino acids other than arginine and valine, whereas the 10.2% protein diet was deficient in methionine, lysine and tryptophan.

Supplementation of the 10.2% protein diet with the deficient amino acids improved the efficiency of protein utilization and additional B vitamins at high or low levels further enhanced the effect. Amino

acid supplementation of the diet also resulted in a greater decrease in liver lipids than occurred with the B vitamins.

Incorporating egg albumen at a 2% level resulted in marked gains in weight. The effects of B-vitamin supplementation were more pronounced with the basal diet than with the diet supplemented with egg albumen. These results were discussed in terms of amino acid requirements and amino acid-vitamin relationships.

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Distribution of Serum Proteins in Biotin-Deficient Rats

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A number of investigators have suggested that there is a relationship between biotin and protein metabolism. Pizzolato and Beard ('45) reported a significant decrease of muscle creatine in biotin-deficient rats. Macleod and Lardy ('49) indicated the function of biotin in protein synthesis. Konikova et al. ('50) observed an increased protein formation when excess biotin was fed to biotin-deficient rats. Kritsman et al. ('53) further observed that a sharp increase of labeled methionine incorporation into tissue protein resulted when normal rats were given small doses of biotin. In a recent study, similar observations were reported by Mistry and Dakshinamurti ('61) in biotin-deficient rats. A biotin deficiency in chicks has been reported to result in sharp decrease in albumin synthesis in liver (Poznanskys, '57).

Biotin is also reported to influence the metabolism and synthesis of aspartic and glutamic acid and deamination of aspartic acid, serine and threonine (Stokes et al., '47; Lichstein, '51; Gothosakar and Sreenivasan, '53). Considering the proposed role of biotin in protein metabolism, it seemed worth while to study whether biotin deficiency would influence the level of total serum protein or any of its electrophoretic components in rats, as similar studies have not been previously reported.

EXPERIMENTAL

Weanling male rats (25 to 30 gm), born to mothers maintained with the laboratory stock diet, were used in the present investigation. Animals that grew well were paired into two groups and housed in individual metabolism cages. Biotin deficiency was induced in one of the pairs by feeding a synthetic diet devoid of the particular vitamin plus avidin of egg white as antivitamin. Avidin, contained in the egg white, forms a biologically inactive complex with biotin making unavailable to animal both the biotin synthesized by the intestinal flora and that taken in with the diet. Control animals were pair-fed with the deficient animal receiving a complete synthetic diet. Two drops of vitamin A and D concentrate[2] were fed to each rat twice a week. The composition of the diets is given in table 1.

TABLE 1
Composition of diets[1]

	Control diet	Biotin-free diet
	gm	*gm*
Alcohol-extracted casein	20	20
Sucrose	60	60
Salt mixture no. 4[2]	4	4
Ground-nut oil	5	5
Autoclaved egg-white	11	—
Raw dried egg-white	—	11

[1] Synthetic vitamins added as supplement to each 100 gm of diet: (in milligrams) thiamine·HCl, 0.2; pyridoxine, 0.25; Ca pantothenate, 2.0; niacin, 5.0; inositol, 10.0; biotin, 0.01; *p*-aminobenzoic acid, 25.0; folic acid, 0.02; choline·HCl, 100.0; vitamin E, 2.3; and vitamin K ,0.21.
[2] Hegsted et al. ('41).

The raw egg white was prepared by separating the white from the yolk and drying it in the sun. For the autoclaved egg white, eggs were autoclaved at 15 pounds pressure for 15 minutes and then the egg white was separated and dried. Heat treatment destroyed the avidin of the egg white.

Within 6 weeks, the animals receiving the biotin-free diet showed the characteristic symptoms of biotin deficiency, consisting of loss of hair, abnormal posture and gait, spectacled eyes and retarded growth. Complete loss of hair was observed after about 8 weeks of the experimental regimen and animals were extremely ir-

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ritable when touched. All control rats developed well and gained weight rapidly. After the eighth week, when the animals developed severe deficiency, food was withdrawn from the cages in the evening, and on the next morning both a biotin-deficient animal and its normal mate were anesthetized with pentobarbital sodium and blood samples withdrawn by cardiac puncture. Total serum protein was estimated by the biuret method (Gornall et al., '49). Different fractions of serum proteins were separated by paper electrophoresis. One hundredth milliliter of undiluted serum was used and electrophoresis continued for 16 hours at 110 volts in barbiturate-barbituric acid buffer of pH 8.6 and ionic strength, 0.05. Staining of paper strips, determination of optimal density curves of the colored zones by densitometry and calculation of amounts of different fractions of serum proteins were the same as described previously (Banerjee and Chaterjee, '57).

RESULTS

The results are shown in tables 2 and 3. The serum protein pattern of biotin-deficient rats was characterized by significant lowering of albumin ($P < 0.01$) and β-globulin fraction ($P < 0.02$). There was a slight decrease in the γ-globulin fraction which was not significant. There was no significant change in the α_1- and α_2-globulin fractions. The total protein of serum was significantly low in the biotin-deficient rats when compared with that of pair-fed normal controls ($P < 0.01$). A slight decrease in A/G ratio was also observed in biotin deficiency.

TABLE 2

Serum protein in rats[1]

Rats	Serum protein
	gm/100 ml serum
Normal	7.76 ± 0.72[2,3]
Biotin-deficient	6.60 ± 0.80[3]

[1] Eight rats used/group.
[2] Standard deviation of the mean.
[3] Significant difference $P < 0.01$.

DISCUSSION

It has been reported that in biotin deficiency there is a decrease in the incorporation of C^{14}-labeled amino acids into liver protein in rats and chickens (Mistry and Dakshinamurty, '61) which implicates this vitamin in the biosynthesis of protein. A similar decrease in albumin synthesis in liver was indicated by Poznanskys ('57) in biotin-deficient chicks. These effects were demonstrated to be related to interference with some of the various reactions involved in the Krebs cycle in which biotin is known to take part, with consequent decrease in the release of adequate energy for the synthesis of protein and nucleic acid. In the present experiments, therefore, the decrease in total serum protein and serum albumin, may be due to diminished synthesis of these components by the liver of biotin-deficient animals.

Total serum protein content in normal control rats has been observed to be apparently higher than previously observed by us in aminopterin-induced folic acid-deficient rats (Rohatgi and Banerjee, '59). This might be the result of the high-protein diet (egg white) being fed to these animals. The electrophoretic pattern of normal se-

TABLE 3

Different fractions of serum protein in rats[1]

Rats	Albumin	Globulins				Albumin globulin ratio
		α_1	α_2	β	γ	
	gm/100 ml serum					
Normal	2.46 ± 0.42[2]	1.28 ± 0.18	0.82 ± 0.08	1.53 ± 0.09	1.67 ± 0.31	0.46
Percentage of total protein	31.70	16.49	10.57	19.72	21.52	
Biotin deficient[3]	1.71 ± 0.37	1.23 ± 0.24	0.88 ± 0.28	1.34 ± 0.16	1.44 ± 4.33	0.35
Percentage of total protein	25.91	18.64	13.33	20.30	21.82	

[1] Eight rats used/group.
[2] Standard deviation of the mean.
[3] The mean values for normal rats differed sufficiently from those of the biotin-deficient to give a P value of < 0.01 for albumin and of < 0.02 for β-globulin in serum.

rum protein is also not similar to that reported previously (Rohatgi and Banerjee, '59), probably due to the high-protein diet. This is in agreement with the observations of Mulgoankar and Sreenivasan ('57).

The changes in different components of plasma proteins in biotin deficiency indicate the interrelationship of biotin nutrition and protein metabolism in the body.

SUMMARY

Different fractions of serum proteins were determined by paper electrophoresis in biotin-deficient and pair-fed normal rats. Total serum protein was significantly low in the deficient animals. There was a significant decrease in albumin and β-globulin with no significant change in α_1-, α_2- and γ-globulin fractions in the serum of biotin-deficient rats when compared with pair-fed normal controls.

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Effect of Age, Sodium Depletion and Sodium Repletion on the Retention of Sodium^{22} by Rats[1]

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Gabriel (1894) first demonstrated that chloride-free bone residue contained large quantities of sodium. Forty years later, the amount of bone sodium in excess of that predictable from the chloride content was defined as "extra" bone sodium (Harrison et al., '36). Since that time, many investigators have adduced further information indicating the presence in the skeleton of about 30 to 40% of the total body sodium. Within recent years, studies with radiosodium (Forbes and Perley, '51; Edelman et al., '54; Davies et al., '52; Bauer, '54) indicate that 30 to 40% of the bone sodium is exchangeable with circulating sodium. Recent reviews (Bergstrom, '56; Nichols and Nichols, '56) on the role of bone sodium in the general sodium economy of the body shed little light on the metabolic behavior of sodium incorporated into the "non-exchangeable" bone sodium pool.

Kellerman ('58) suggested the possibility of very long-term retention of bone-deposited Na^{22} in rats. Results of previous experiments at this Laboratory (Richmond, '58) showed the presence of a long retention component in the whole body retention pattern of Na^{22} administered to 5 species of animals. The data suggested that human subjects retained less than 1% of the administered activity with a biological half-life (T_b) of approximately 460 days. Miller and associates ('57) at Leeds also detected the presence of this long-lived component in a human subject with the aid of a high pressure ionization chamber and, subsequently, recommended the limited use of Na^{22} in clinical investigations. This component, which is thought to represent radiosodium tenaciously bound in bone, has been detected only with the aid of whole body counting systems. The contribution to the total integrated radiation dose from radiosodium deposited in the skeleton is by no means insignificant as at least 10% of the total disintegrations occurring within the body of man after Na^{22} administration is associated with the long-lived component.

This experiment was designed to investigate the effects of age, sodium depletion, and sodium repletion on the long-term retention pattern of radiosodium as an attempt to learn more about the turnover and metabolic activity of the slowly exchanging bone sodium pool.

METHODS AND MATERIALS

Thirty-six male Sprague-Dawley rats of two different ages were used. Twelve animals 30 days of age (mean weight, 106 gm) and 12 animals 86 days of age (mean weight, 292 gm) were divided into groups of 6 animals each. Two groups (one of each age) were fed ground commercial laboratory chow,[3] and the other two were fed a commercially supplied low sodium diet.[4] An additional 12 animals were used for tissue distribution studies. Members of this group were given Na^{22} intraperitoneally and killed at intervals during the experiment to determine the Na^{22} content of the entire skeleton as a function of time after injection. Bones and teeth were removed from the skinned animals after autoclaving for about one hour. All animals were housed individually in metabolism cages and allowed free access to diet

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[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

[4] Nutritional Biochemicals Corporation, Cleveland.

and water throughout the experimental period.

After the rats had been fed the respective diets for a three-week acclimatization period, each rat was given 0.4 μc of Na^{22} as the chloride by intraperitoneal injection. Thirty minutes later, the gamma ray activity of each animal was measured by whole body counting in a 4 π liquid scintillation counter (Schuch, '60). A standard consisting of an aqueous solution of $Na^{22}Cl$ in a polyethylene bottle, 5 in. long and 2.25 in. in diameter, was measured with each group of rats. Animals and standards were measured at appropriate intervals over a 163-day period. Forty days after Na^{22} administration, the two groups supplied with the low sodium diet were transferred to a diet of ground commercial laboratory chow[5] and allowed to replete their body sodium for the remainder of the experiment. The biological retention of the Na^{22} was then calculated by the following expression:

$$\text{Biological retention} = \frac{\left(\frac{\text{Animal count rate}}{\text{Standard count rate}}\right)_t}{\left(\frac{\text{Animal count rate}}{\text{Standard count rate}}\right)_o}$$

in which the subscripts t and o refer to the times after Na^{22} administration. This expression eliminates the effect of radioactive decay and corrects for minor day-to-day fluctuations in the counting system.

RESULTS AND DISCUSSION

In figure 1 is shown the mean whole body biological retention values as a function of time for all 4 groups of animals. Of the animals fed the commercial laboratory chow throughout the experimental period (lower curves), the younger animals lost radiosodium at a faster rate initially and retained more of the administered dose at later times than the adult animals.

Data represented by the upper curves in figure 1 show that both age groups retained Na^{22} tenaciously during the period of sodium restriction. During this period, younger rats appeared to retain more of the radiosodium than the adult rats. Of the administered activity, only 4 and 16%, respectively, were lost from the young and old animals at the end of the depletion

[5] See footnote 3.

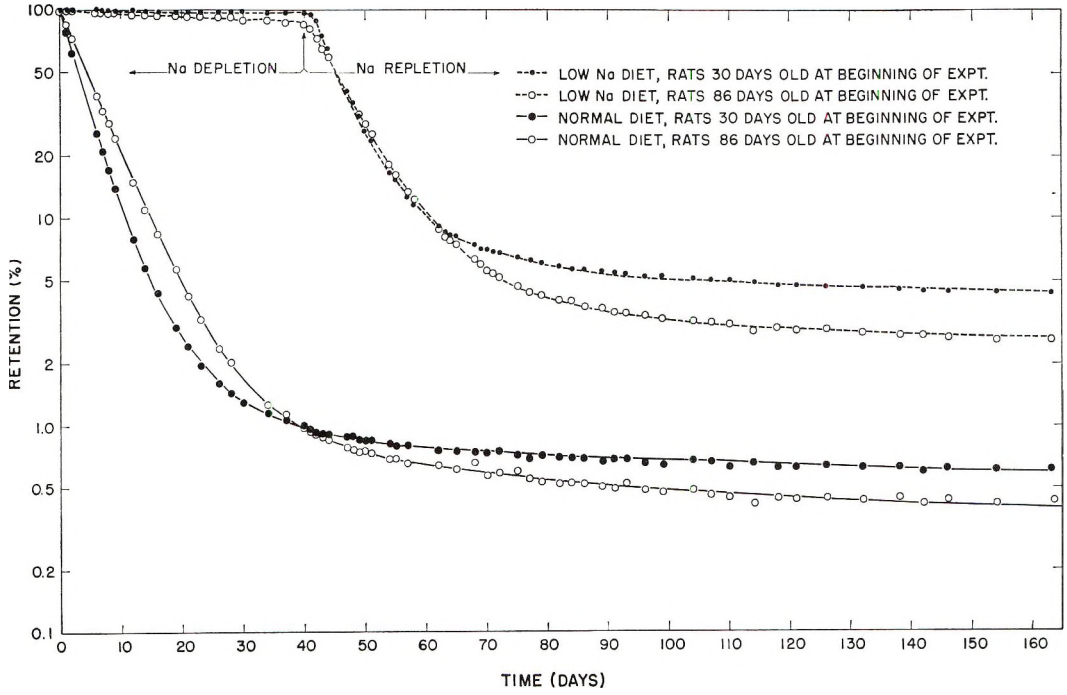

Fig. 1 Effect of age, sodium depletion and sodium repletion on the retention of Na^{22} by rats. Dashed lines are eye fitted to the data points, whereas the solid lines represent the best fit retention functions as calculated by the IBM 704 computer.

period. Burch et al., ('49) showed that retention of Na^{22} in rats varied inversely with the level of sodium in the diet. Hine et al. ('60) demonstrated a similar change in Na^{22} retention as a function of sodium intake in one human subject. Loss of Na^{22} from the body was drastically reduced, whereas the sodium intake was about 1 mEq per day, and noticeably increased when the normal diet was supplemented with sodium.

After the change to commercial laboratory chow on day 40, however, sodium repletion began and both groups lost Na^{22} at an accelerated rate. The younger animals appeared to lose the radiosodium more rapidly during the initial phase of the repletion period than adult animals did. The younger animals, however, ultimately incorporated more radiosodium into the slowest changing component.

The most striking feature of figure 1 is the large difference between the amounts of Na^{22} retained by the normal and repleted groups at long times after administration. Younger rats in both normal and repleted groups retained more of the initial Na^{22} burden than adult rats. One might expect relatively larger amounts of Na^{22} to deposit in the skeleton during sodium repletion. Neuman and Neuman ('57) postulated that when a patient suffering from a prolonged dietary sodium depletion is supplied with an adequate sodium intake, the sodium-poor bone crystals should soak up large quantities of the sodium ion. These workers believe that because the composition of the bone crystal is not fixed, it merely reflects by ion exchange the composition of the fluid to which it has been exposed. The data in figure 1 appear to support this postulate. Presumably, the slowest changing component represents that fraction of the bone sodium commonly called "non-exchangeable" bone sodium. Relatively large amounts of Na^{22} may be bound in this fraction during sodium repletion because skeletal sodium, which was mobilized during the period of restricted sodium intake, is being replaced.

The retention data for both the young and adult age groups maintained with the normal sodium diet were reduced to exponential components by an IBM 704 computer programmed to perform iterative least square calculations. Coding procedures require the retention values (Y) at each time (t) and estimates of the intercept and rate parameters of the function. Each value of Y is weighted statistically by a factor proportional to 1/Y. This procedure avoids the subjectivity inherent to eye fitting a line to the experimental data and, by iteration, assures that best fit criteria are equally applicable to all components simultaneously.

The biological retention functions for the lower curves in figure 1 are of the form,

$$R_t = \sum_{i}^{3} \left[a_i e^{-k_i t} \right]$$

where R_t is the biological retention as percentage of the administered activity at any day t; a and k are the intercept and rate constant for each term; and e is the base of natural logarithms. The values for a and k which best describe the retention data out to 163 days are:

$$R_t = 91.14\, e^{-0.24715\, t} + 7.52\, e^{-0.09196\, t} + 0.88\, e^{-0.00247\, t}$$

and

$$R_t = 98.60\, e^{-0.16341\, t} + 1.19\, e^{-0.03511\, t} + 0.59\, e^{-0.00257\, t}$$

for young and adult groups, respectively. These equations are represented by the solid lines drawn in figure 1. The last term of each equation shows that 0.88% of the injected dose was retained by the young animals with a T_b of 281 days, whereas 0.59% of the dose was retained with a T_b of 269 days in the older animals. These components should not be ignored when estimating total radiation dose, as they account for about 38 and 22%, respectively, of the total area under the effective retention function (namely, as the result of physical decay and biological loss of Na^{22}) from t_o to t_∞.

Figure 1 indicates that the radiation dose delivered to animals of either age group while maintained with the restricted sodium intake would be considerably greater than that delivered to animals kept on the normal diet. This is true also during the repletion phase, as much more of the administered Na^{22} is associated with the slowly changing component.

The parameters describing biological retention in the adult animals maintained

with the ground commercial laboratory chow were used to estimate the compartmental partition of the total body sodium pool. The rate constant k is presumed to be the ratio of sodium turnover to pool size; the intercept value a is the fraction of the administered Na^{22} deposited in or associated with each pool at t_o. The fraction of the total sodium pool represented by each component can then be obtained by dividing the value for each relative pool size by the sum of the relative pool sizes. These values are shown in table 1. It is possible that components 1, 2, and 3 represent extracellular, intracellular, and "extra bone" sodium, respectively. If this is true, the total bone sodium pool would be about 38% of the total body sodium pool. To obtain this estimate, we assume that 30% of the total bone sodium is exchangeable and not included as part of component 3. Presumably, the freely exchangeable bone sodium is lost as part of the extracellular sodium pool (component 1) at a constant rate of 0.1634 day^{-1}. The value of 3.9% for component 2 is reasonably similar to the values of 5% given by Bergstrom ('55) as the size of the intracellular sodium pool for adult rats and the approximately 3% given by Edelman ('59) for man.

In table 2 a similar analysis for the young animals is summarized. Component 3, assumed to represent the "extra" bone sodium component, accounts for about 44% of the total body sodium pool. If one again assumes that "extra" bone sodium accounts for 70% of all bone sodium, it would appear that the total bone sodium is about 63% of the total body sodium pool. But the fraction of the total bone sodium that is freely exchangeable probably is not the same as in the adult rat.

Results of the tissue distribution studies suggested that on days 64, 105, 169, and

TABLE 1

Body sodium pools of adult rats[1] calculated from biological retention parameters

Component	Na^{22} in pool at t_o a[2]	Sodium turnover rate k[3]	Relative pool size with each turnover rate a/k	Percentage of total pool with each turnover rate
				% of total sodium pool
1	98.60	0.16341	599.72	69.5
2	1.19	0.03511	33.89	3.9
3	0.59	0.00257	229.72	26.6
Total	100.38	—	863.33	100.0

[1] Eighty-six days old at Na^{22} administration.
[2] a = % of injected Na^{22} in component at t_o.
[3] $k = \frac{0.693}{T_b}$ day^{-1}.

TABLE 2

Body sodium pools of young rats[1] calculated from biological retention parameters

Component	Na^{22} in pool at t_o a[2]	Sodium turnover rate k[3]	Relative pool size with each turnover rate a/k	Percentage of total pool with each turnover rate
				% of total pool
1	91.14	0.24715	368.76	45.71
2	7.52	0.09196	81.77	10.13
3	0.88	0.00247	356.28	44.16
Total	99.54	—	806.81	100.00

[1] Thirty days old at Na^{22} administration.
[2] a = % of injected Na^{22} in component at t_o.
[3] $k = \frac{0.693}{T_b}$ day^{-1}.

237 after injection, 75 to 85% of the whole body Na^{22} activity was in the skeletal tissues. The remainder, which appeared in the skinned eviscerated carcass, may be an artifactual result of the autoclaving procedure used to separate bone from muscle. Also, the small amount of Na^{22} in the animals at these sampling times precludes any precise measurement of any activity that might be in tissues other than bone.

SUMMARY

A 4 π liquid scintillation counter was used to measure the whole body retention of $Na^{22}Cl$ for 173 days in young and adult rats fed either a ground commercial laboratory chow or a low sodium diet. Whole body retention of Na^{22} was measured also during a sodium repletion phase. Results of these studies indicated that radiosodium retention can be described by the sum of three exponential components. A slowly exchanging bone component with a biological half-time of 270 to 280 days was observed. Age-dependent differences in the retention patterns were noted.

Sodium^{22} turnover was extremely slow in the sodium depleted animals. Only 4 and 16% of the administered activity were lost from the young and adult animals, respectively, at the end of 40 days. The amount of radiosodium bound in the slowly exchanging bone component of the sodium-repleted animals was larger by a factor of about 10 when compared with animals maintained with the normal diet. The slowly exchanging component, characterized by the 270- to 280-day biological half-time, was thought to represent the bone sodium pool that is not freely exchangeable with circulating sodium. This pool accounted for 44% of the total body sodium pool in the young rats and 27% in the adult rats.

This work points out the effect of the nutritional state of an organism on the biological retention of a radionuclide and, therefore, on the radiation dose delivered to the animal. These implications should be considered by workers, especially clinicians, using Na^{22}.

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The Mechanism of Gossypol Detoxification by Ruminant Animals[1]

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It has been known for many years that cottonseed meal containing free gossypol produces toxic symptoms if fed to swine (Withers and Carruth, '15, '18) or chicks (Couch et al., '55; Rigdon et al., '58) at significant levels, and that gossypol has been demonstrated to be toxic to other animals (Withers and Carruth, '18; Schwartze, '24; Eagle, '50). But it has also been demonstrated that relatively large amounts of gossypol contained in cottonseed meal may be fed to ruminant animals with no sign of toxicity (Cranford, '10; Jones et al., '41; Ramsey and Miles, '53).

The mechanism whereby gossypol is detoxified by the ruminant animal has remained unknown. Since most differences in metabolism between ruminant and nonruminant animals may be traced to the activity of rumen microorganisms, the possibility was considered that these organisms might also act upon gossypol to destroy it in some manner.

Previous work in this laboratory has shown that dietary polyunsaturated fatty acids may be hydrogenated by rumen liquor,[2] or in the rumen *in vivo* (Reiser and Reddy, '56). This suggested that gossypol might also be reduced in the highly reducing environment of the rumen.

EXPERIMENTAL

Preparation of soluble gossypol. About 50 mg of crystalline gossypol[3] were dissolved in 15 ml of alcohol. Twenty milliliters of glycerol were added and the alcohol evaporated on a steam bath under reduced pressure. The product is a clear sol which may be mixed with water in any proportion without precipitation.

Determination of free and bound gossypol. Gossypol assays, free and bound, were determined by the method of Pons and Hoffpauir ('57).

Determination of free ε-amino group. This assay was made by the method of Baliga et al. ('59).

Preparation of rumen liquor. Rumen liquor was obtained from freshly slaughtered beefs, then strained through cheese cloth and used as soon as possible.

Aerobic and anaerobic incubation. To determine whether anaerobic or aerobic organisms have a different influence on gossypol, 3.5 ml of the strained liquor were mixed with 1.25 ml of gossypol sol. In one set of experiments 0.5 ml of 4% glucose and in another set 0.5 ml of 95% alcohol were added as a source of energy. The results of the study are given in table 1. There was no loss of total gossypol, but the free gossypol was significantly reduced, indicating a binding effect but no destruction. There was probably no significant difference between the aerobic and anaerobic cultures, bringing into question bacterial participation in the free gossypol disappearance. Nevertheless glucose apparently stimulated more activity than alcohol. However, different rumen liquors were used and there was no control run without glucose or alcohol.

Effect of energy source. The influence of the nature of the added energy source was further studied by anaerobic incubation. The results (table 2) do not support the superior activity of glucose. This sug-

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[2] Reiser, R. 1951 Hydrogenation of polyunsaturated fatty acids by the ruminant. Federation Proc., *10*: 236 (abstract).

[3] Gossypol acetate was sent to us from the Southern Utilization Research and Development Division, USDA, New Orleans, Louisiana by Dr. T. H. Hopper whose kindness we gratefully acknowledge.

TABLE 1

Effect of aerobic and anaerobic incubation of rumen fluid and gossypol on the disappearance of total and free gossypol

Incubation conditions[1]	Disappearance of gossypol			
	Test 1[2,3]		Test 2[3,4]	
	Free	Total	Free	Total
	%	%	%	%
Anaerobic	62 ± 4.9[5]	0	29 ± 2.6	0
Aerobic	75 ± 2.1	0	36 ± 4.1	0

[1] Incubation at 37°C for 48 hours.
[2] Test 1. Rumen liquor, 3.5 ml; 4% glucose, 0.5 ml; and gossypol solution, 1.25 ml.
[3] Each value is the result of 4 replicate tests.
[4] Test 2. Rumen liquor, 3.5 ml; 95% alcohol, 0.5 ml; and gossypol solution, 1.25 ml.
[5] Standard deviation.

TABLE 2

Effect of incubating gossypol and rumen liquor anaerobically with glucose or alcohol

Media[1] no.	Disappearance of gossypol			
	Test 1[2]		Test 2[2]	
	Free	Total	Free	Total
	%	%	%	%
1[3]	85 ± 9.0[4]	0	80 ± 5.2	0
2[5]	70 ± 1.8	0	33 ± 4.4	0
3[6]	81 ± 4.6	0	54 ± 10	0

[1] Incubation at 37°C for 48 hours.
[2] Each value is the result of 4 replicate tests.
[3] Medium no. 1. Rumen liquor, 5 ml; gossypol solution, 0.2 ml.
[4] Standard deviation.
[5] Medium no. 2. Rumen liquor, 5 ml; gossypol solution, 0.2 ml; and 2% glucose, 2 ml.
[6] Medium no. 3. Rumen liquor, 5 ml; gossypol solution, 0.2 ml; and 95% alcohol, 1 ml.

TABLE 3

Effect of centrifugation of rumen fluid at various speeds on the formation of the bound gossypol

Centrifugation speed (20 min.)	Disappearance of free gossypol				
	Test 1[1]	Test 2[2]	Test 3[3]	Test 4[4]	Test 5[5]
rpm	%	%	%	%	%
None		30 ± 0[6]	36 ± 2.2	67 ± 9.1	59 ± 0
500	49				
1,000		33 ± 2.5	29 ± 2.4		
2,000	48	48 ± 2.5	39 ± 8.3		
2,500				67 ± 3	59 ± 0
4,500	58				
5,000		30 ± 5			
6,000		45 ± 5	31 ± 3.1	69 ± 6	59 ± 0
6,500	55				
10,000				64 ± 0	
10,500					59 ± 0

[1] Test 1. Rumen liquor, 10 ml; gossypol solution, 0.2 ml; incubated aerobically at 37°C for 24 hours; one tube at each speed.
[2] Test 2. Rumen liquor, 5 ml; gossypol solution, 0.2 ml; and 1% glucose, 2 ml; incubated anaerobically at 37° C for 48 hours, in duplicate.
[3] Test 3. Rumen liquor, 5 ml and gossypol solution, 0.2 ml; incubated anaerobically at 37°C for 48 hours, in triplicate.
[4] Test 4. Rumen liquor, 5 ml and gossypol solution, 0.4 ml; heated for 20 minutes at 70°C in a water bath, in duplicate.
[5] Same as test 4, except that a different sample of rumen fluid was used, in duplicate.
[6] Standard deviation.

gests that the reaction is probably not due to a reduction of the gossypol to a colorless derivative by bacteria and again suggests that the disappearance of free gossypol is not due to microbial action.

Activity of centrifuged rumen liquor. To determine whether the gossypol is bound by bacteria or by soluble protein, samples of rumen liquor were centrifuged at various speeds up to 10,500 rpm and then incubated with gossypol sols for 48 hours at 37°C. The results (table 3) show clearly that centrifugation had no influence on the percentage of gossypol that was bound. Although bacteria are difficult to remove completely from rumen liquor by centrifugation, there are relatively few remaining in the supernatant after 20 minutes at 10,500 rpm, and one should expect a graded decrease in numbers from uncentrifuged liquor. If the gossypol had been bound by the bacteria one should expect a decrease in the degree of binding in the centrifuged specimens. Because no decrease occurred, the tentative conclusion was made that the gossypol was bound by soluble protein.

Nonbacterial nature of the reaction. A second test to determine whether the gossypol is bound by rumen bacteria or soluble protein was made by adding gossypol sol to the liquor and immediately immersing in a hot water bath at 70°C for 10, 20, 30, or 60 minutes.

The results (table 4) show clearly that the binding effect cannot be due to the metabolic action of bacteria because the reaction was completed at 70°C in 10 minutes or less. The reaction is thus chemical.

TABLE 4

Effect of heating gossypol-rumen fluid solutions for varying periods of time at 70°C on the disappearance of total and free gossypol

Heating period	Disappearance of gossypol[1,2]	
	Free	Total
minutes		
10	81 ± 1.7[3]	0
20	79 ± 2.5	0
30	80 ± 4.4	0
60	82 ± 7.4	0

[1] Determined in triplicate.
[2] The test solutions contained 0.4 ml of gossypol solution and 5 ml of rumen fluid.
[3] Standard deviation.

Comparison of bacterial incubation and hot water temperatures. To obtain some comparison between the hot water bath and incubation reactions, 10-ml samples of rumen liquor were mixed with 4 ml of gossypol sol and either held at 70°C for 20 minutes, aerobically, or incubated at 37°C for 48 hours anaerobically. Duplicate tests gave identical results, the 70°C reaction binding somewhat more gossypol than the 37°C incubation reactions (table 5).

Relation between binding of free gossypol and disappearance of lysine ε-amino groups. A final test of whether the gossypol is bound by rumen liquor protein is the simultaneous disappearance of free gossypol and lysine ε-amino groups. Samples of rumen liquor were incubated at 37°C for 48 hours with the ratios of rumen to gossypol sol of 5 to 0.4 and 10 to 0.4. One sample was heated at 70°C for 20 minutes with the ratio of rumen to gossypol sol of 5 to 0.4. The milligrams of free gossypol and lysine ε-amino groups, that disappeared were determined (table 6). The mole ratio of bound gossypol to bound lysine was 1:2 in three of the 4 tests.

Influence of proteolytic enzymes on the stability of rumen liquor bound gossypol. To test the effect of digestion upon the stability of the lysine-gossypol complex, 5 ml of rumen liquor were incubated for 24 hours at 37°C with 0.2 ml of gossypol sol. One milligram of the enzymes was added and incubation continued for an additional 24 hours. The percentage of free gossypol that disappeared was then determined. The addition of enzymes had no influence on the gossypol binding activity of the liquor (table 7).

TABLE 5

Comparison of bound gossypol formation under different types of incubation

Types of incubation	Disappearance of gossypol[1,2]	
	Free	Total
	%	%
Aerobic water bath at 70°C for 20 minutes	75 ± 0[3]	0
Anaerobic at 37°C for 48 hours	64 ± 0	0

[1] In duplicate.
[2] The test samples contained 10 ml of rumen liquor and 4 ml of gossypol solution.
[3] Standard deviation.

TABLE 6

The simultaneous disappearance of the free ε-amino group of lysine and of free gossypol during incubation of rumen fluid-gossypol solution

Test[1] no.	Disappearance of free lysine ε-amino group		Disappearance of free gossypol		Mole ratio of bound gossypol : bound lysine
	mg	*moles*	*mg*	*moles*	
1[2]	0.53 ± 0.08[3]	0.0034	1.48 ± 0.11	0.0031	1 : 1.2
2[4]	1.24 ± 0.26	0.0084	2.28 ± 0.16	0.0044	1 : 1.9
3[5]	0.98 ± 0.045	0.0067	1.58 ± 0	0.0031	1 : 2.1
4[6]	0.99 ± 0.0045	0.0068	1.75 ± 0	0.0033	1 : 2.0

[1] All tests were run in duplicate.
[2] Test 1. Five milliliters of rumen liquor incubated anaerobically with 0.4 ml of gossypol-glycerol solution at 37°C for 48 hours.
[3] Standard deviation.
[4] Test 2. Ten milliliters of rumen liquor incubated anaerobically with 0.8 ml of gossypol solution at 37°C for 48 hours.
[5] Test 3. Ten milliliters of rumen liquor incubated anaerobically with 0.4 ml of gossypol solution at 37°C for 48 hours.
[6] Test 4. Ten milliliters of rumen liquor incubated aerobically with 0.4 ml of gossypol solution at 70°C in water bath for 20 minutes.

TABLE 7

Effect of proteolytic enzymes on the disappearance of free gossypol bound in rumen fluid-gossypol solution[1]

Enzymes added	% disappearance of free gossypol
Test 1[2]	
No enzyme	32 ± 4.1[3]
Pancreatin	38 ± 2.5
Rhozyme-6	38 ± 6.1
Rhozyme-pf	25 ± 0
Test 2[4]	
No enzyme	79 ± 1.6
Pepsin	84 ± 2.9
Trypsin	77 ± 4.9

[1] The basic incubation medium in each tube was 5 ml of rumen fluid and 0.2 ml of gossypol solution. After incubation for 24 hours at 37°C, one milligram of the enzyme was added to the medium and incubation continued for an additional 24 hours.
[2] In duplicate.
[3] Standard deviation.
[4] In triplicate.

SUMMARY AND CONCLUSION

The indifference of the degree of gossypol binding of rumen liquor to aerobic or anaerobic incubation, high or low temperature, centrifugation or proteolytic enzymes, and the simultaneous disappearance of two moles of lysine ε-amino groups to each mole of gossypol, demonstrates convincingly that the mechanism of ruminant detoxification of gossypol is by binding to soluble proteins, and that the bond is permanent during protein digestion.

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Cholesteryl Ester Fatty Acid Patterns of Plasma, Atheromata and Livers of Cholesterol-fed Rabbits[1]

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Cholesterol-fed rabbits develop atheromatous infiltrations of the aortic intima. The mechanism by which this occurs is still unknown, but it is generally assumed that the disturbance of the cholesterol metabolism following excessive dietary cholesterol supply is closely related to the formation of atheromata. The most striking characteristics of this disturbance are, besides the occurrence of atheromata, the accumulation of cholesteryl esters in the plasma, and fatty infiltration and enlargement of the liver.

The abnormally increased plasma lipids are a possible source of the lipids observed in the atheromata. Schwenk and Stevens ('60) showed that almost all of the cholesterol of these lesions is derived from the plasma; phospholipids on the other hand are synthesized in the lesions and do not originate from the plasma (Zilversmit et al., '54). The origin of the fatty acids of the cholesteryl esters (CEFA) of the atheromata is unknown, and the comparison of CEFA patterns of the plasma with those of the lesions may possibly disclose evidence for or against the hypothesis that the latter are directly derived from the circulation, as is the case for cholesterol itself.

For this purpose we have attempted to define the essential alterations of plasma CEFA patterns occurring in rabbits fed a 1% cholesterol diet, and compared the CEFA patterns of the aortic atheromata and livers of the same animals with those of the plasma.

MATERIALS AND METHODS

Seven rabbits were fed a diet consisting of 96% of commercial rabbit chow, 3% of peanut oil and 1% of cholesterol[2] for 8 months. The animals were then killed and their aortas dissected. The intima of each aorta was severely infiltrated with atheromatous lesions, so that only very limited zones of normal intima were noted between the arch and the orifices of the renal arteries. The lesions could easily be peeled off the underlying tissue, and each aorta yielded sufficient material for separate analyses. The tissue was weighed and ground fine in a mortar with about 5 gm of pure quartz grains. The resulting powder was stored at −15°C until analyzed.

The livers of the rabbits were also removed and ground up in a mixer; aliquots were further fragmented in Potter tubes. All samples, and also plasma taken before death, were stored at −15°C until analyzed.

The lipids of aliquots of aorta or liver were extracted with 20 volumes of methylal:methanol 4:1 at room temperature. After filtration, a second extraction was performed with boiling chloroform under reflux, and both extracts were combined. The analysis using the grinding procedure of aortic tissue just described was compared with the analysis after the method described by Böttcher et al. ('60) and good agreement was obtained with respect to the yield of lipids and fatty acid patterns.

Aliquots of the extract obtained as outlined were evaporated, taken up in light petroleum ether and chromatographed on silicic acid columns. The cholesteryl esters were eluted with 1% anhydrous ether in light petroleum ether (Hirsch and Ahrens, '58). A known quantity of heptadecanoic acid was added to the eluate, which was then evaporated. The residue

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[2] Merck, Darmstadt.

TABLE 1

CEFA patterns of cholesterol-fed rabbits[1]

	Controls	Cholesterol-fed			Zilversmit's data[3]	
No. of animals	21[2]	7			3	
	Plasma	Plasma	Aortic intima	Liver	Plasma	Aortic intima
	Fatty acid pattern (% of total CEFA)					
Myristic acid	0.4	1.1	2.9	1.7	0.9	1.3
Palmitic acid	18.2	15.8 ± 3.5[4]	14.4 ± 2.5	13.8 ± 2.9	17.8	13.5
Palmitoleic acid	2.5	3.1	2.5	3.2	5.1	4.5
Stearic acid	3.4	4.6 ± 1.0	4.0 ± 1.0	3.4 ± 1.2	4.3	2.9
Oleic acid	33.6	50.2 ± 3.0	57.4 ± 6.9	63.2 ± 6.5	44.2	54.9
Linoleic acid	39.2	24.2 ± 2.9	18.5 ± 2.8	13.5 ± 3.1	24.3	16.1
	Total CEFA concentration (*mg per 100 ml plasma or per 100 gm of wet tissue*)					
	20.8	605	2,890	3,251	—	—
	Oleic/linoleic acid ratio					
	0.9	2.1	3.1	4.7	1.8	3.4

[1] These figures do not include the acids with 20 and more carbon atoms.
[2] Three pools of 7 normal plasma samples.
[3] Averaged from table 2 of Zilversmit ('61) (cholesterol-fed rabbits: 1 gm daily for 16 weeks).
[4] Standard deviation.

was methanolyzed with methanol and paratoluene sulphonic acid as a catalyst; in these conditions the internal standard is converted to methyl heptadecanoate. The methyl esters so obtained were extracted with petroleum ether (Stoffel et al., '59) and chromatographed in a beta ray argon gas chromatograph[3] using Reoplex 400 as stationary phase, at 160°C. The absolute amount of CEFA could be computed from the surface area of the peaks obtained for each fatty acid and that of the known quantity of heptadecanoic acid.

CEFA patterns of normal plasma were obtained by taking the average result of the analysis of three pools of 7 normal rabbit plasma samples.

RESULTS

The plasma CEFA patterns of normal and hypercholesterolemic rabbits are represented in table 1, as well as those of the atheromata and the livers of the latter group of animals. The results of Zilversmit et al. ('61) which were obtained in a similar experiment, are also indicated. Only the major components are included.

The plasma CEFA patterns of cholesterol-fed rabbits were distinctly different from normal patterns in their high percentage of oleic acid and low value of linoleic acid. The absolute amount of circulating cholesteryl linoleate, however, was far above normal values, due to 30-fold increase of plasma cholesteryl esters in these rabbits. The ratio of oleate/linoleate in cholesteryl esters increased from 0.9 in the controls to 2.1 in the hypercholesterolemic rabbits; this latter value corresponds well with that derived from Zilversmit's data.

Apart from these alterations no gross differences were noted between the control and experimental groups. The sum of the saturated acids was not markedly changed, and the sum of the monoene and diene acids also appeared fairly stable.

The CEFA of the atheromata were similar to those of the plasma, but the ratio of oleate/linoleate was still higher, which again confirms the data of Zilversmit. It can be calculated that the concentration of linoleate, expressed as milligrams per 100 gm of fresh tissue, is 3.7 times higher in the lesions than in the plasma (expressed as mg per 100 ml), but the concentration of oleate is 5.5 times higher.

In the liver the prevalence of oleate in the CEFA patterns was even more pronounced and the concentration of linoleate was very low. Again, no other striking

[3] W. G. Pye Ltd., Cambridge, England.

differences were noted with either normal or hypercholesterolemic plasma CEFA patterns, or with those of the atheromata.

The sum of the relative concentrations of oleate and linoleate appears to be about the same in each pattern, and close to 75% of the total CEFA.

DISCUSSION

Dietary administration of cholesterol to rabbits results in a typical disturbance of CEFA patterns. In plasma, as well as in atheromatous tissue and liver, the oleate/linoleate ratio is substantially increased. Data corroborating this observation can be calculated from other studies (Zilversmit et al., '61). The precise metabolic step that accounts for this effect is not apparent from our results.

The dissimilarity between the CEFA patterns of plasma and atheromata excludes the likelihood that the cholesteryl esters accumulate in the aortic intima by a simple random deposition of plasma cholesteryl esters. If, indeed, the plasma is the source of the CEFA of the atheromata, one must accept either that there exists, for oleate, a greater permeability of the aortic wall than for linoleate, or that the inherent metabolism of the aortic intima reshapes the CEFA patterns of the deposited lipids. Human early atheromatous lesions also display an elevated ratio of cholesteryl oleate/linoleate as compared with normal adjacent intima of the same aortas (Nelson et al., '61). A local disturbance of CEFA metabolism resembling that observed in cholesterol-fed rabbits, thus seems to be closely related to the formation of human atheromata.

The high concentrations of cholesteryl oleate and low concentrations of linoleate in plasma or tissue containing increased quantities of cholesteryl esters could be explained by the hypothesis that the cholesterol-fed rabbit requires an abnormally high amount of linoleic acid to assimilate the large quantities of exogenous cholesterol. Hence it will be interesting to investigate whether the alteration of CEFA patterns could be suppressed by supplementing the cholesterol diet with an excessive amount of linoleic acid. Because in man a diet rich in linoleic acid has been found to increase the percentage of this fatty acid in the plasma CEFA (Okey et al., '60), the influence of such a diet on atherogenesis deserves further investigation.

SUMMARY

The cholesteryl esterified fatty acids (CEFA) of plasma, liver and aortic lesions of cholesterol-fed rabbits were analyzed by gas-liquid chromatography. The patterns of these acids were characterized chiefly by a high percentage of oleic acid, especially in the aortic intima and in the liver, where the oleic-linoleic acid ratio was increased up to 3.1 and 4.7, respectively, against 2.1 in the plasma and 0.9 in the plasma of normal rabbits. In each instance the sum of the relative concentrations of oleic and linoleic acids represented approximately 75% of the total CEFA.

The dissimilarity between the CEFA patterns of plasma and atheromata excludes the likelihood that the cholesteryl esters accumulate in the aortic intima by a simple random deposition of the plasma cholesteryl esters.

The CEFA patterns of rabbit atheromata closely resemble those reported by other authors in human early atheromatous lesions.

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