THE NUTRITION OF SALMONOID FISHES

I. CHEMICAL AND HISTOLOGICAL STUDIES OF WILD AND DOMESTIC FISH

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The salmon fishing industry of the Pacific Coast is dependent on the survival and propagation of 5 species of salmon which spawn in rivers of that portion of the North American continent extending from California to Alaska. The development of these rivers for power, irrigation, flood control and other projects has drastically reduced the natural spawning areas available to salmon. To prevent the extinction of these fish and the concurrent losses to the fishing industry, various State and Federal agencies have initiated a major program of artificial propagation. This area of animal husbandry has received little attention from nutritional workers and there is a paucity of information on the nutritional requirements of salmon. To supply this essential information the U. S. Fish and Wildlife Service established the Salmon Nutrition Laboratory at Cook, Washington in 1952. This report represents the first of a series which will be presented from the laboratory.

One problem is unique to the field of salmon nutrition: the young must be raised for variable periods in a completely domestic and artificial environment, then released in a wild environment to compete with wild fish and grow to maturity. The most important criterion of successful propagation, therefore, is their ability to survive in the wild habitat. There is considerable evidence that the usual criteria of nutritional

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studies, growth or weight gains, may not be good indices of ability to survive. The body composition of the young fish, however, appears to be of essential importance. Many studies have shown that survival and condition of artificially propagated fish are inferior in comparison with wild salmonids (Klak, '41; Williamson and Schneberger, '43; Schuck, '45, '48; Miller, '52, '54). In conjunction with this problem, several workers have reported that hatchery fish are morphologically different from wild fish. Lower erythrocyte counts and hemoglobin levels (Marsh, '02; Marsh and Gorham, '06), abnormal fatty degeneration of the liver (Hewitt, '37a, b), and extensive fatty degeneration of the pancreas (Hess, '35; Donaldson, '39) have been described. A chemical study on brook trout showed definite differences in body composition between wild and hatchery-reared fish (Phillips et al., '55) and objective organoleptic tests gave significantly higher scores to wild trout (Baeder et al., '48).

Many of the above studies involved only a single species or small numbers which were possibly not typical of hatchery fish in general since there is a great variation in hatchery techniques and diets. The present study was designed, therefore, as an essential preliminary to future nutritional studies to determine if chemical and anatomical differences are a common characteristic of wild and hatchery salmonids and if so, to investigate the reasons for these differences and to establish standards of body composition of naturally propagated fish.

METHODS AND MATERIALS

During a two-year collection period, 150 samples were obtained of wild and hatchery salmonids representing 9 species of trout and salmon found in Oregon and Washington. The species were chinook salmon *(Oncorhynchus tshawytscha)*, coho salmon *(O. kisutch)*, sockeye salmon *(O. nerka)*, chum salmon *(O. keta)*, pink salmon *(O. gorbuscha)*, rainbow trout *(Salmo gairdneri)*, brown trout *(S. trutta)*, cutthroat

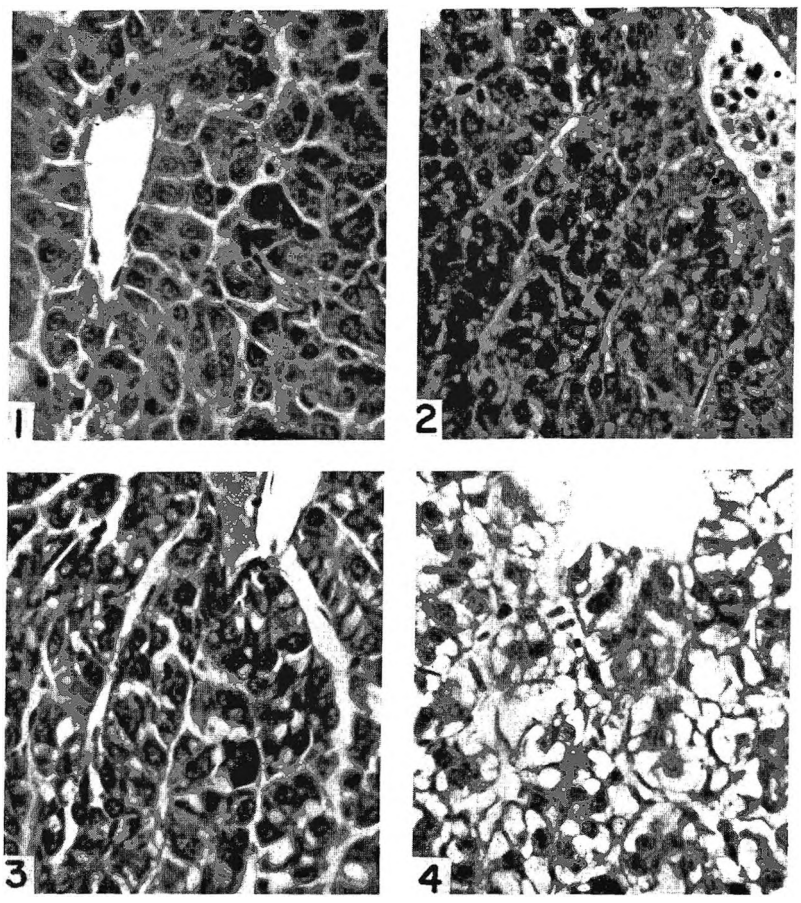
trout *(S. clarki),* and brook trout *(Salvelinus fontinalis).* When available, fingerlings less than one year of age were compared with fish more than one year of age to determine if differences became more accentuated with time. Since some of the salmon species migrate to the ocean immediately after hatching, it was not possible to make age comparisons for these species. In other cases, the difficulty in obtaining wild samples eliminated some comparisons. The chinook samples were separated into the fall run and spring run varieties, since the natural life histories of the two types are markedly different. The rainbow samples were similarly separated into the rainbow and steelhead varieties.

Each sample was treated as follows for later histological and chemical studies:

Histological. Fish were fixed in Bouin's solution for 24 hours and stored in 65% ethyl alcohol. A portion of each sample was fixed and stored in 10% neutral formalin for subsequent frozen sections and fat stains. Each fish was numbered, the caudal peduncle severed and a blood smear made for future hematological studies. Routine paraffin sections were made from 5 fish of each collection. Longitudinal whole sections were made from small specimens under two inches in length. Larger specimens were dissected and sections prepared from the eye, gill, heart, skin and muscle, anterior kidney, posterior kidney, liver, spleen, stomach and caecal section of the intestinal tract. Hematoxylin and eosin were used routinely and, where indicated, Giemsa, Gram, Schiff, glycogen and fat stains were utilized.

The tissue sections were evaluated for (1) liver fat, (2) pancreatic and visceral fat, (3) indications of disease, (4) degree of parasitism, and (5) presence of ceroid. The incidence of each variable was rated by assigning a value of from 1 to 4, 1 indicating a negative observation. The degrees of liver fatty infiltration are illustrated in figures 1 to 4. The degrees of fatty deposition in and around the pancreas are shown in figures 5 to 8.

Chemical. Each sample was preserved for chemical analysis by two methods. From 150 to 500 gm, depending on the size of the fish, were homogenized in a Waring Blendor with 2 parts by weight of 2:1 chloroform:methanol solution



Figures 1–4 Degrees of liver fatty infiltration. H and E stain. × 430.

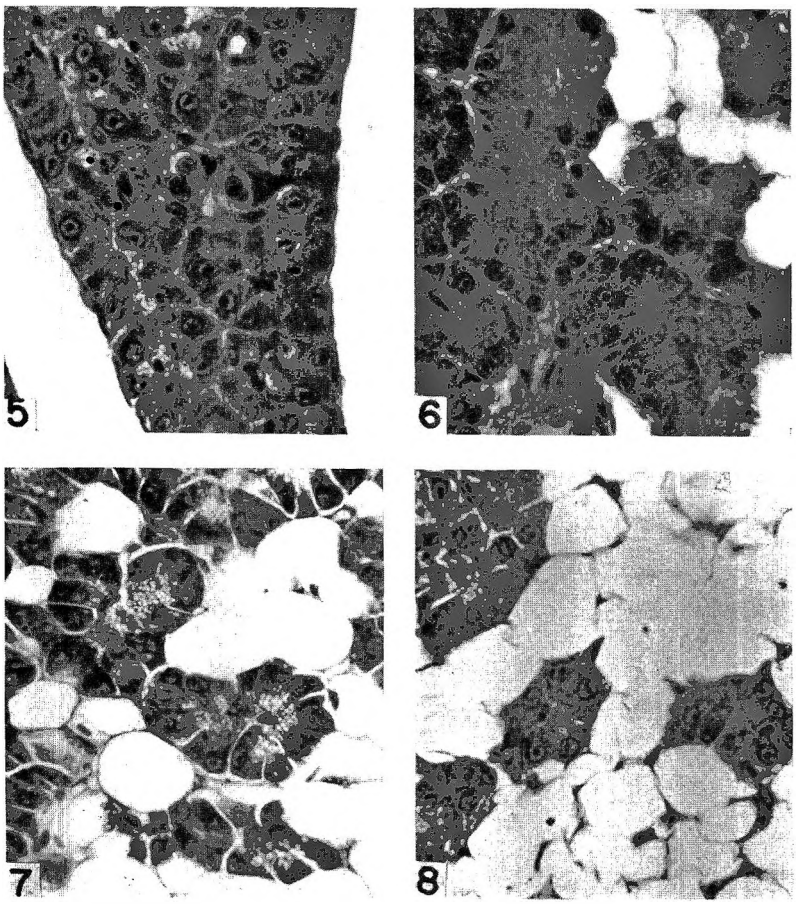
Fig. 1 (Type 1) Non-fatty liver of adult wild cutthroat trout. Compare with pancreas in figure 8 from same fish.

Fig. 2 (Type 2) Slightly fatty liver of wild cutthroat trout.

Fig. 3 (Type 3) Moderately fatty liver of hatchery steelhead trout.

Fig. 4 (Type 3) Extremely fatty liver of young hatchery fall chinook salmon. Compare with pancreas in figure 5 from same fish.

and stored in pint jars. Samples of equal size were quick-frozen on dry ice for transportation to the laboratory where they were transferred to quart jars and stored under nitrogen at —10°C. For analysis, the samples were re-homogenized in a Waring Blendor to give a uniform suspension from which



Figures 5–8 Degrees of fatty deposition in and around the pancreatic tissue. H and E stain. × 430.

Fig. 5 (Type 1) Non-fatty pancreas of young hatchery fall chinook salmon.
Fig. 6 (Type 2) Slightly fatty pancreas of hatchery rainbow trout
Fig. 7 (Type 3) Moderately fatty pancreas of adult wild cutthroat trout.
Fig. 8 (Type 4) Extremely fatty pancreas of adult wild cutthroat trout.

duplicate 10 ml samples were pipetted into (a) crucibles for ash determination, (b) aluminum weighing dishes for protein analysis, and (c) 50 ml mixing cylinders for lipid analysis. Ash and protein samples were dried to constant weight at 60°C. *in vacuo,* the average of the 4 being used as the dry weight for the lipid sample. Sample weights varied less than 1%. In some instances, notably with a low-fat sample, difficulty was encountered in obtaining a uniform suspension. In this case, a fresh homogenate was prepared using a frozen fish sample and just sufficient chloroform: methanol solvent (1 to 1.5 vol.) to give a uniform suspension. Ash and protein (Kjeldahl nitrogen) determinations were carried out by official AOAC methods. In order to obtain a lipid extract suitable for subsequent analytical work, the method of Folch et al. ('51) was employed. This, essentially, involves the extraction of wet tissue with a relatively large volume of chloroform: methanol (2:1) solvent, freeing the filtered extract of non-lipid contaminants by allowing it to remain in contact with a large volume of water and subsequently evaporating the solvent to obtain the weight of lipid. The method was modified as follows: the homogenate contained in a 50 ml mixing cylinder was diluted to 50 ml with the solvent mixture, thoroughly mixed and filtered with the filtrate being caught in a 100 ml beaker placed in the bottom of a liter beaker nearly full of distilled water; the cylinder and the material remaining on the filter paper were washed with several small portions of solvent mixture, and the washings collected with the original extract in the beaker; after standing overnight, the aqueous layer was siphoned off, the fluff layer dissolved with methanol and the solvent allowed to evaporate; the extract was dried to constant weight at 60°C. *in vacuo,* the lipid dissolved and transferred to a suitable container for subsequent analysis and the beaker re-weighed with the loss in weight reported as total lipid. The homogenized and frozen samples gave values in close agreement.

The data from both the histological and chemical samples were compared statistically by utilizing the "t" test to compare the group means of each species since there was no basis for pairing the individual samples (Snedecor, '46).

RESULTS AND DISCUSSION

Histological. The anatomical comparisons are presented in table 1. With few exceptions, the livers and viscera of hatchery fish were rated as more fatty than wild fish. However, the two groups were by no means at opposite extremes. In evaluating these sections, the observer did not know whether the tissue was of wild or hatchery origin. After studying the sections from each collection, a group "guess" was made. Only 69% of both groups were identified correctly. It is apparent, therefore, that the two groups were not histologically distinct.

There was little difference in disease incidence as indicated by inflammatory or necrotic lesions. The degree of parasitism was consistently higher in wild fish. This consisted of intestinal organisms, encysted trematode metacercariae, gill parasites, and miscellaneous sporozoans (to be reported separately).

The wild fish definitely showed a higher occurrence of ceroid deposits. Ceroid has previously been observed in diseased wild and hatchery fish (Wood and Yasutake, '56) but only a single report is known of its occurrence in nonmoribund wild fish (Pickford, '53). In mammals, the acid-fast, sudanophilic pigment is closely associated with the accumulation of unsaturated fatty acids and a relative lack of biological antioxidants (Casselman, '51, '53). The fatty acids of wild fish have been reported as more highly unsaturated than those of hatchery fish with the two values approaching each other after hatchery fish have spent a prolonged time in a wild environment (Phillips et al., '49, '50). The more frequent occurrence of ceroid in wild fish is, no doubt, correlated with their more highly unsaturated fatty acids. Whether the pigment indicates a relative or absolute deficiency

TABLE 1

Histological comparison of hatchery-reared and wild salmonids [1]
The variables are rated on an ascending scale from 1 to 4

SPECIES	AGE [2]	NO. SAMPLES	LIVER FAT	P	PANCREAS FAT	P	DISEASE	P	PARASITISM	P	CEROID	P
	yrs.			P [3]		P		P		P		P
F. Chinook	0											
H.		6	3.3	< 0.05	1.5	n.s.	2.0	n.s.	1.7	n.s.	1.0	n.s.
W.		4	2.0		1.5		2.0		2.3		1.0	
S. Chinook	0											
H.		5	2.4	n.s. [4]	2.0	< 0.01	2.0	n.s.	2.0	n.s.	1.0	n.s.
W.		6	2.3		1.3		2.0		2.2		1.0	
S. Chinook	1											
H.		1	2	.. [5]	3	..	2	..	1	..	2	..
W.		1	1		2		2		2		1	
Sockeye	1											
H.		2	2.0	..	3.0	..	2.0	..	1.0	..	1.5	..
W.		1	1		1		2		2		2	
Silver	0											
H.		6	3.5	< 0.08	1.3	n.s.	1.8	n.s.	1.5	n.s.	1.0	n.s.
W.		8	2.9		1.1		1.8		1.6		1.2	
Silver	1											
H.		6	3.0	n.s.	3.2	< 0.01	2.7	n.s.	2.2	n.s.	1.5	< 0.01
W.		6	2.3		1.8		2.3		2.5		2.5	
Pink	0											
H.		1	2	..	1	..	1	..	1	..	1	..
Chum	0											
W.		2	3.0	..	1.0	..	1.0	..	1.0	..	1.0	..
Steelhead	1											
H.		7	2.9	< 0.02	3.3	< 0.06	2.0	n.s.	2.4	n.s.	1.1	n.s.
W.		6	1.8		2.5		1.5		2.2		1.5	
Rainbow	0											
H.		5	2.6	..	2.2	..	2.0	..	1.8	..	1.2	..
Rainbow	1											
H.		6	2.2	n.s.	2.8	n.s.	1.8	n.s.	1.8	n.s.	1.0	n.s.
W.		5	2.6		2.6		2.0		2.2		1.4	
Cutthroat	1											
H.		6	3.0	n.s.	2.6	n.s.	2.5	n.s.	1.7	n.s.	1.0	< 0.02
W.		8	2.4		2.3		2.8		1.4		1.6	
Brown	1											
H.		1	4	..	4	..	2	..	1	..	1	..
W.		2	2.0		3.0		2.0		2.0		2.0	
Brook	1											
H.		3	4.0	..	3.0	..	2.3	..	1.7	..	1.0	..
W.		3	3.0		2.7		2.0		2.3		1.7	
Total												
H.		55	2.8	< 0.01	2.5	< 0.01	2.1	n.s.	1.7	< 0.02	1.1	< 0.01
W.		52	2.4		1.9		2.0		2.0		1.5	

[1] Each value is a group mean.
[2] Zero age means less than one year.
[3] Probability of a higher value of "t" with the hypothesis that the group means are equal.
[4] The "t" value is not significant: hypothesis accepted.
[5] Sample too small for valid statistical comparison.

TABLE 2

Proximate analysis of wild and hatchery-reared salmonids [1]

SPECIES	AGE [2]	NO. SAMPLES	PROTEIN		LIPID		ASH	
	yrs.		%	P [3]	%	P	%	P
F. Chinook	0							
H.		15	70.2	< 0.01	19.9	< 0.02	9.5	n.s.[4]
W.		2	77.7		15.1		10.0	
S. Chinook	0							
H.		5	72.1	< 0.02	18.8	< 0.03	10.6	n.s.
W.		4	77.9		13.7		11.1	
S. Chinook	1							
H.		1	71.4	..	20.8	..	11.3	..
Silver	0							
H.		9	70.2	< 0.01	19.0	< 0.01	10.1	< 0.06
W.		7	78.0		12.3		11.6	
Silver	1							
H.		11	64.3	< 0.01	26.6	< 0.01	10.2	< 0.05
W.		4	76.6		11.2		13.3	
Blueback	1							
H.		2	63.8	.. [5]	28.7	..	9.7	..
W.		1	77.6		14.8		10.6	
Pink	0							
H.		1	70.0	..	17.1	..	11.7	..
Chum	0							
W.		1	76.6	..	18.1	..	8.7	..
Steelhead	1							
H.		9	64.5	< 0.01	26.3	< 0.01	10.1	< 0.01
W.		6	77.0		11.5		13.2	
Rainbow	0							
H.		6	67.7	..	22.0	..	10.5	..
Rainbow	1							
H.		18	67.9	n.s.[4]	24.0	< 0.04	10.6	< 0.01
W.		6	69.6		18.6		13.2	
Cutthroat	0							
H.		1	74.0	..	15.1	..	11.1	..
Cutthroat	1							
H.		5	69.6	< 0.01	21.6	< 0.01	10.4	< 0.01
W.		8	76.6		11.1		14.5	
Brown	1							
H.		1	68.0	.. [5]	24.8	..	9.3	..
W.		1	72.8		12.3		13.1	
Brook	1							
H		3	66.6	.. [5]	20.1	..	11.4	..
W.		2	71.7		16.7		10.7	
Total								
H.		87	68.1	< 0.01	22.5	< 0.01	9.7	< 0.01
W.		42	75.8		13.4		12.3	

[1] Each value is a group mean.

[2] Zero age means less than one year.

[3] Probability of a higher value of "t" with the hypothesis that the group means are equal.

[4] The "t" value is not significant: hypothesis accepted.

[5] Sample too small for valid statistical comparison.

of biological antioxidants, or is due to other factors remains to be elucidated.

Chemical. The proximate analysis values are listed in table 2. Without exception, the protein levels were lower and the lipid values were higher in hatchery-reared fish. With only one exception (brook trout), the ash levels were lower in domestic fish.

A comparison between the two groups at different ages (table 3) was particularly informative. Even in the younger

TABLE 3

Changes in hatchery-reared and wild salmonids with age [1]

	NO.	PROTEIN		LIPID		ASH		NO.	LIVER FAT [3]		PANCREAS FAT [3]	
		%	%ch.[2]	%	%ch.	%	%ch.			%ch.		%ch.
Hatchery												
0 Year	37	70.1	−4.6	19.7	+21.8	10.1	+ 5.0	23	2.9	0.0	1.7	+76.5
1+ Year	42	66.9		24.0		10.6		32	2.9		3.0	
Wild												
0 Year	12	77.6	−3.9	13.8	− 4.3	11.1	+22.5	20	2.6	−15.4	1.2	+91.7
1+ Year	26	74.6		13.2		13.6		32	2.2		2.3	

[1] Each value is a group mean.

[2] The figure in the %ch. column indicates the per cent change in body composition between fish less than one year old and more than one year old.

[3] The incidence of liver and pancreatic fat is rated on an ascending scale from 1 to 4.

groups, the differences in composition were well established. In fish over one year in age, the protein values remained fairly constant, but the fat content of hatchery fish increased 22% compared to a slight drop in wild fish. The increase in ash content of wild fish was 4 times that of hatchery fish.

The increased lipid in hatchery fish was accompanied by a marked increase in pancreatic or visceral fat. A similar increase of visceral fat occurred in wild fish, although neither liver fat nor total body lipid showed a corresponding change. In hatchery fish, the correlation coefficient for microscopically visible fat in the liver and pancreas changed from $r = -0.05$ in young fish, to $r = 0.68$ in the older group. In contrast,

the values for wild fish changed from $r = 0$ to $r = -0.12$. An explanation for this puzzling lack of correlation in all wild fish and in younger hatchery fish may lie in the poorly developed pancreatic systems of these animals with their minimal number of islets (Hess, '35) and consequential poor metabolism of carbohydrate diets (Phillips et al., '48). In the absence of high-fat diets, therefore, general fat deposition may result only with difficulty. In hatchery fish, even at a very young age, liver fat is consistently high with a subsequent increase in visceral depots and total body lipid. In contrast, the visceral depots of wild fish increase slowly with no preceding liver infiltration or simultaneous increase in total content. There is, therefore, considerable indication that high liver fat and high pancreatic fat are not characteristically seen together. The converse of this in older hatchery fish carries considerable significance.

The histological and chemical data offer conclusive evidence that hatchery-reared fish are, practically without exception, markedly different in body composition from wild fish of the same species and age. It further appears that these differences become recognizable after a relatively short period of hatchery environment and become progressively more emphatic as length of hatchery rearing increases.

There is little difference in disease incidence between the two groups and this factor is discounted as a significant variable. Parasitism is definitely more pronounced in wild fish, and it is conceivable that this could be responsible for the lower fat content. However, the increased protein and ash levels of wild fish do not support such a contention. That emphatic differences in nutrition exist between the two groups is obvious and the data strongly suggest that artificial diets are a major cause of the differences observed in body composition. The precise effect of these differences on the ability of fish to survive in a wild habitat is an unanswered question of considerable importance which will be the subject of continuing studies.

SUMMARY

Salmonids reared under artificial conditions show marked consistent differences in body composition in comparison with wild salmonids. Protein and mineral levels are lower and lipid values are higher in hatchery fish than in wild fish. As the period of artificial rearing is increased, these differences become more extreme. In hatchery fish, there is generally more microscopically visible fat in the liver and viscera than in these organs in wild fish although extremes are seen in both groups. In young hatchery fish and in wild fish, there is no correlation between fat deposition in the liver and in the pancreas, but in older hatchery fish, both of these organs are fatty and there is a simultaneous increase in total body lipid. In wild fish, ceroid deposition is greater, suggesting that the fatty acids are more highly unsaturated. There is little difference in disease incidence between the two groups, but parasitism is more pronounced in wild fish.

The significant variables between wild and artificially produced fish appear to be limited to diet and environment. From the factors discussed above, diet alone is probably the most important single factor in producing the changes observed in body composition.

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LITERATURE CITED

BAEDER, H. A., P. I. TOCK AND A. S. HAZZARD 1948 A comparison of the palatability of hatchery-reared and wild brook trout. Trans. Am. Fish. Soc., *75* (1945): 181.

CASSELMAN, W. G. B. 1951 The *in vitro* preparation and histochemical properties of substances resembling ceroid. J. Exp. Med., *94:* 549.

———— 1953 Factors influencing the formation of ceroid in the livers of choline-deficient rats. I. Dietary fats. Biochem. et Biophys., Acta, *11:* 445.

DONALDSON, L. R. 1939 Experimental studies in the nutrition of the chinook salmon with special reference to histological changes in the pancreas. University of Washington (Seattle) Ph.D. Thesis.

FOLCH, J. I. ASCOLI, M. LEES, J. A. MEATH AND F. N. LEBARON 1951 Lipid extraction from brain tissue. J. Biol. Chem., *191:* 833.

HESS, W. N. 1935 Reduction of the islets of Langerhans in the pancreas of fish by means of diet, overeating, and lack of exercise. J. Exp. Zool., *70*(2): 187.

HEWITT, E. R. 1937a Some recent work on fatty livers in trout. Prog. Fish-Cult., *27:* 11.

———— 1937b Behavior of hatchery trout in streams. Ibid., *28:* 33.

KLAK, G. E. 1941 The condition of brook trout and rainbow trout from four eastern streams. Trans. Am. Fish. Soc., *70* (1940): 282.

MARSH, M. C. 1902 Hemoglobin estimations and blood counts in fishes. Wash. Med. Ann., *1:* 397.

MARSH, M. C., AND F. P. GORHAM 1906 Hemoglobin and blood counts in fishes in health and disease: a review. Sci. (N.S.) 23, (S91), 666pp.

MILLER, R. B. 1952 Survival of hatchery-reared cutthroat trout in an Alberta stream. Trans. Am. Fsh. Soc., *81* (1951): 35.

———— 1954 Comparative survival of wild and hatchery-reared cutthroat trout in a stream. Ibid., *83* (1953): 120.

PHILLIPS, A. M., A. V. TUNISON AND D. R. BROCKWAY 1948 The utilization of carbohydrates by trout. N. Y. Cons. Dept. Fish. Res. Bull no. 11, 44 pp.

PHILLIPS, A. M., D. R. BROCKWAY, M. BRYANT, E. O. RODGERS AND J. N. MAXWELL 1949 The nutrition of trout. Ibid., no. 13: 26.

PHILLIPS, A. M., D. R. BROCKWAY, A. J. J. KOLB AND J. N. MAXWELL 1950 The nutrition of trout. Ibid., no. 14: 20.

PHILLIPS, A. M., F. E. LOVELACE, H. A. PODOLIAK, D. R. BROCKWAY AND G. C. BALZER 1956 The nutrition of trout. Ibid., no. 19: 57.

PICKFORD, G. W. 1953 Fish endocrinology. A study of the hypophysectomized male killifish, *Fundulus heteroclitus* (Linn). Bull. Bingham Oceanographic Coll., *14*(2): 5.

SCHUCK, H. A. 1945 Survival, population density, growth, and movement of the wild brown trout in Crystal Creek. Trans. Am. Fish. Soc., *73* (1943): 209.

SCHUCK, H. A. 1948 Survival of hatchery trout in streams and possible methods of improving quality of hatchery trout. Prog. Fish-Cult., *10*(1): 3.

SNEDECOR, G. W. 1946 Statistical Methods. Iowa State College Press, Ames, Iowa. 4th ed.

WILLIAMSON, L. O., AND E. SCHNEBERGER 1943 The results of planting legal-sized trout in the Deerskin River, Vilas County, Wisconsin. Trans. Am. Fish. Soc., *72* (1942): 92.

WOOD, E. M., AND W. T. YASUTAKE 1956 Ceroid in fish. Am. J. Path., *32*(3): 591.

THE NUTRITION OF SALMONOID FISHES

II. STUDIES ON PRODUCTION DIETS

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The body composition of salmonids raised in hatcheries is markedly different, both chemically and histologically, from that of wild fish (Wood et al., '57). Differences between artificial and natural diets were suggested as important causative factors. Several workers have compared specific hatchery diets to a wide array of wild organisms which form the components of wild diets (Embody and Gordon '24; Schaeperclaus, '33; Phillips et al., '54, '56). There have been few descriptive reports, however, in this field of animal husbandry and on the variations which exist in the composition and compounding of production diets and the effects of these variations on body composition of hatchery fish.

The purpose of this paper is to describe the diets which are now used to raise salmonoid fishes in the Pacific Coast states, to present the variation which exists in the proximate analysis of these diets and to relate the diet composition to the body composition of the hatchery product. The factors instrumental in producing the extreme differences in body composition between wild and hatchery fish were of particular interest.

METHODS

Samples of hatchery salmonids representing 9 species were collected from 40 State and Federal fish hatcheries in Oregon and Washington. Diet samples were taken at each station,

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sealed in pint jars, quick-frozen and stored at —10°C. until analyzed. In each case, the diet samples were taken at random from much larger batches of production diet prepared for routine feeding. At some stations, the same diet was fed to several species of fish so that a total of 69 diet samples was fed to 87 fish samples. Proximate analysis was completed on the diet samples as previously described for fish (Wood et al., '57) with the following modification in the sample preparation: sufficient frozen sample for all determinations was removed from the jar with a high-speed drill which yielded a finely divided, uniform sample; per cent moisture was determined on protein and ash samples to obtain the dry weight of the sample for lipid determinations; extraction of lipid was done by homogenizing the wet sample in a Waring Blendor (micro attachment) using approximately 20 volumes of solvent mixture; the slurry was then filtered into a small beaker contained in a large beaker of water as before and the Blendor cup and residue were washed with several small portions of solvent; the remainder of the procedure remained unchanged. The values for body composition of wild and hatchery fish were taken from the previous study.

DIETS

Salmonid nutrition has, by necessity, relied more on available foodstuffs than on known nutritional requirements. Early classified as a carnivore by its food habits and relatively short digestive tract, the salmonoid fish was the first principal user of slaughter house by-products. Hatchery fish are almost traditionally thought of as "liver fed" although this commodity forms a minor part by volume of modern production diets. With increasing competition for these products from the fur farming industry and pet food manufacturers, fish raisers have gradually been forced to other feeds. Three types of foodstuffs now form the major part of salmonoid diets: slaughter house offal, marine scrap fish and fish canning by-products, and various types of dry feeds (fish meals, cereals, distillery by-products). In general, fish diets in the

eastern part of the country utilize more dry feeds while in the west, diets consist of more fishery products.

In compounding fish diets, considerable emphasis has been placed by various investigators on simulating the composition of wild diets in respect to the basic nutrients, fat, protein, ash and carbohydrate. Average proximate analysis values of wild organisms from three sources are listed in table 1. The natural diet of salmonids, thus, is characterized as high in protein and moderately low in fat and carbohydrate.

The components of the 69 production diets referred to in this paper are listed in table 2. The range of each component in different diets is also listed, as is the percentage of diets in

TABLE 1

Proximate analysis of invertebrate organisms composing typical wild diets

	PROTEIN	LIPID	ASH	CARBOHYDRATE		SOURCE
	%	%	%	N-free ext. %	Fiber %	
	64.6	12.4	9.6	13.5		Phillips et al. ('54)[1]
	48.7	15.5	9.9	17.9	8.0	Embody and Gordon ('24)
	59.0	9.9	12.5	8.6	12.7	Schaeperclaus ('33)[2]
Av.	57.4	12.6	10.7	13.3	10.4	

[1] Determined by difference. All values converted to dry weight.
[2] All values converted to dry weight.

which each ingredient appears. The diets generally contained many different ingredients. The meat portion of a typical one contained salmon viscera with eggs 21, smelt heads and viscera 21, beef spleen 0.5, beef tripe 0.5, pork spleen 0.5, beef lungs 1.0 and horse meat 0.5. In addition the following dry feeds were included: dry skim milk 18, wheat middlings 18, cottonseed meal 18, yeast 1.0 and salt 0.5. Mixtures of this type are commonly resorted to in compounding fish diets in an attempt to avoid deficiencies of possible growth factors. In the absence of basic knowledge on the nutritional requirements involved, the value of such mixtures appears questionable.

It is apparent that fish-production diets show extreme variation in the type, level and diversity of their ingredients The

TABLE 2

Components of 69 salmonid production diets: the range in different diets and the percentage of diet occurrence

	LEVEL, RANGE	DIET OCCURRENCE
	%	%
Slaughterhouse by-products:		
Liver		
Beef	6–100	67
Hog	33–75	22
Lamb	3–4	6
Horse	33	3
Tripe		
Beef	0.5–10	12
Sheep	6	1
Spleen		
Beef	0.5–80	33
Hog	0.5–25	16
Beef lungs	1–25	16
Beef lips	10–13	3
Mixed offal	100	4
Horse meat	0.5–35	7
Beef meat	5–10	4
Fish products:		
Salmon viscera	20–85	64
Salmon carcass	30–63	38
Salmon eggs	5–40	13
Sole	43–50	10
Canned salmon	17–25	4
Carp	44	1
Halibut	2–70	4
Tuna viscera	10	1
Hake	14	1
Smelt heads and viscera	20	1
Dry feeds:		
Meat meal	5–25	19
Yeast	1–10	13
Fish meal	10–25	10
Cottonseed meal	3–12.5	12
Wheat shorts	5–12.5	13
Dry milk	10–12.5	9
Distillers' solubles	5	1
Salt (NaCl)	0.5–2	52

effect of this lack of standardization is evident in the analysis which follows.

RESULTS AND DISCUSSION

The proximate analysis data are summarized in table 3. Marked variation characterized each element of the diet but

TABLE 3

Proximate analysis of 69 salmonid production diets, of the domestic fish receiving these diets, and of comparable wild fish

	PROTEIN	LIPID	ASH	CARBOHYDRATE
	%	%	%	%
No. samples:				
Diets — 69				
Hatchery fish — 87				
Wild fish — 42				
Range:				
Diets	20.0–76.6	10.2–79.5	1.6–24.3	0–35.8
Hatchery fish	55.7–79.6	11.9–38.1	7.4–13.8	0–7.9
Wild fish	63.7–81.1	7.6–25.0	8.4–16.2	0–3.4
Mean:				
Diets	62.4	23.0	10.0	4.8
Hatchery fish	68.1	22.5	9.7	0.6
Wild fish	75.8	13.4	12.3	0.4
Standard deviation:				
Diets	8.97	8.98	5.22	6.90
Hatchery fish	4.90	5.38	2.69	1.42
Wild fish	4.01	3.99	2.75	0.91
Coef. variation, %:				
Diets	14.4	39.1	52.1	144 [1]
Hatchery fish	7.2	23.9	27.8	237
Wild fish	5.3	29.7	22.3	227

[1] Many zero values for carbohydrate lead to these high coefficients of variation.

there was good agreement between the mean values of the diets and the mean values of the hatchery fish. The variation in the body composition of wild fish was quite similar to that of hatchery fish, but the mean values were markedly different.

The great diversity in diet composition suggested that the extremes in body composition might be related to specific diet components. Accordingly, the diets were divided into 4 groups

on the following basis: (1) 50% or more of salmon products; (2) 50% or more of fish products (a portion of which was often salmon); (3) less than 50% fish; and (4) no fish products. The proximate analysis of the diets in each category and of the fish fed these diets is summarized in table 4. The very marked similarity of mean values of both diets and of fish in all classifications indicates that the extreme values in body composition of hatchery fish are not related to any specific diet components, but that production diets, in general, are at considerable variance with the foods which salmonids would consume naturally. As far as major nutrients are concerned,

TABLE 4

Proximate analysis of 69 production diets grouped according to content of fish products and of the fish fed these diets [1]

COMPOSITION	LIPID		PROTEIN		ASH	
	Diet	Fish	Diet	Fish	Diet	Fish
	%	%	%	%	%	%
Salmon, 50% or more	21.5	22.8	64.6	67.2	10.4	10.2
Fish, 50% or more	23.2	21.4	60.7	67.6	16.1	10.2
Fish, less than 50%	22.6	22.5	62.8	68.6	7.0	10.4
No fish	19.3	20.0	60.9	70.8	8.9	10.2

[1] All values are means.

these diets differed quantitatively from wild diets most significantly in higher fat and in lower carbohydrate values.

The data indicate that the body composition of hatchery fish was altered by the composition of the hatchery diet in so far as the proximate analysis values are concerned. The lower level of protein deposition in hatchery fish suggests that the protein component of the diet was possibly inferior in biological value to that of wild diets. This is not unexpected considering the vast differences which exist between hatchery food components and the largely invertebrate organisms of the natural salmonid diet. Protein imbalances may also be important contributing factors to the extremely fatty condition of hatchery fish. Although the protein component was qualitatively different in the diets containing varying amounts of fish or none,

this difference failed to be reflected in an increased protein component in the fish eating the diets. These data indicate, however, that the lower protein levels of hatchery fish are to some extent relative values which are depressed by the high lipid values.

The discrepancy of carbohydrate content between hatchery and natural diets is partially due to the more efficient extraction method for lipid which resulted in low carbohydrate values (determined by difference) for the hatchery diets. In any event, carbohydrates apparently play only a minor role in the composition of either group of fish although they may enhance the formation of fat. The poorly developed system in fish for handling carbohydrates makes this an unlikely possibility. The high mean fat level in the hatchery diet would in itself promote the fatty condition observed. The mean lipid value of diets and fish were remarkably close. However, there was a low correlation ($r = 0.23$) between the fat content of individual diets and the fish consuming them. This indicated that variables other than diet composition might be present. The most obvious of these were a function of different hatchery facilities and of different types of hatchery management. In the first category, such factors as volume and temperature of water were known to fluctuate widely. In the latter group were different methods in the mechanics of feeding, particularly in the level of feeding. If variables of this type were important factors, one would expect that single hatcheries raising more than one species might contribute a disproportionate number of extreme values in body composition. Accordingly, the hatcheries producing the two highest and the two lowest lipid values for one species were compared to hatcheries producing similar extreme values for other species The results were quite significant with 15% of the total hatcheries producing 50% of the extreme values, either high or low. A further subdivision revealed that 4 hatcheries produced 9 of the 16 highest values and two hatcheries produced 5 of the 16 lowest values.

Even with these extreme values there was a low correlation ($r = 0.20$) between fat content of the diet and of the fish. The diet ingredients were apparently not the critical variable as 38% had less than 50% or no fish while 62% had more than 50% fish. If constant factors such as water volume or temperature were the controlling influences, one would expect a hatchery to produce either high extremes or low extremes in all species, but not a combination of the two. Actually, three of 9 hatcheries representing the extreme values produced both high and low values, thus indicating that more variable factors were also operative.

The determination of optimum level of feeding is a major problem almost unique in the field of fish husbandry. Since growth rate depends on water temperature and on size of fish, a series of tables has been prepared relating feeding level to these two variables (Deuel et al., '42). However, the accurate measurement of fish size is often not feasible in large hatchery operations where several hundred thousand individuals may be subdivided into 50 or 100 lots. As a result, much feeding is done by "rule of thumb" and often depends upon how much a group of fish will consume without undue waste. Consequently, conditions exist which are conducive for extreme variability in feeding level from one hatchery to another. The low correlation between diet and fish composition and the preponderance of extreme values in certain hatcheries indicates that overfeeding is probably an important factor in the high fat content of hatchery fish.

Little experimental work exists which will permit an evaluation of the impact of such changes in body composition on the ability of an animal to survive in a wild habitat. Certainly, however, in a survival test between an obese human and a well-conditioned athlete, logic supports the chances of the athlete. The analogy is not a good one, but it is sufficiently accurate to show clearly the need for experimental evaluation of the hatchery product in terms of its body composition. The obese individual would definitely be inferior to the normal in such factors as stamina and endurance. Hoar and co-workers

('49, '52) have shown, in addition, that the ability of the goldfish to resist low as well as high temperatures may be changed by feeding diets containing different fats in high concentrations. Here the effect is apparently related to the different degree of unsaturation of the fatty acids. The physiology of hatchery fish and their ability to survive are doubtlessly affected by similar changes imposed by production diets.

SUMMARY

In 69 production diets fed to hatchery salmonids, there was a wide variation in the ingredients of the diets and in their content of protein, fat, carbohydrate and ash. There was a close correlation between the body composition of hatchery-raised fish and the composition of the diets fed. The data indicated, however, that differences in hatchery management, such as level of feeding, were also important contributing factors in producing the observed body composition of hatchery fish. Wild fish had a markedly different body composition characterized by a much lower level of fat and relatively higher protein and mineral content

The effect on survival of differences in body composition between wild and hatchery fish is discussed. There is a clearly defined need for additional research on the nutritional requirements of salmonids, the relationship between body composition and ability to survive, and for quality control in hatchery nutrition.

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LITERATURE CITED

DEUEL, C. R., D. C. HASKELL AND A. V. TUNISON 1942 The New York State fish hatchery feeding chart, revised ed., Fish. Res. Bull. No. 3, N. Y. Cons. Dept., Albany, New York.

EMBODY, G. C., AND M. GORDON 1924 A comparative study of natural and artificial foods of brook trout. Trans. Am. Fish. Soc., *54:* 185.

HOAR, W. S., AND J. E. C. DORCHESTER 1949 The effect of dietary fat on the heat tolerance of goldfish *(Carassius auratus)*. Can. J. Res., D, *27:* 85.

HOAR, W. S., AND M. K. COTTLE 1952 Dietary fat and temperature tolerance of goldfish. Can. J. Zool., *30:* 41.

PHILLIPS, A. M., R. S. NIELSEN AND D. R. BROCKWAY 1954 A comparison of hatchery diets and natural food. Prog. Fish-Cult., *16:* 153.

PHILLIPS, A. M., F. E. LOVELACE, H. R. PODOLIAK, D. R. BROCKWAY AND G. C. BALZER 1956 The nutrition of trout. N. Y. Cons. Dept., Fish. Res. Bull. No. 19: 57.

SCHAEPERCLAUS, W. 1933 Textbook of pond culture. Tr. by F. Hund. U. S. Fish and Wildl. Serv., Fish Leaflet 311, 260 pp.

WOOD, E. M., W. T. YASUTAKE, A. N. WOODALL AND J. E. HALVER 1957 The nutrition of salmonoid fishes. I. Chemical and histological studies of wild and domestic fish. J. Nutrition, *61:* 465.

INFLUENCE OF MINERAL INTAKE ON BONE DENSITY IN HUMANS AND IN RATS

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For the past 20 years indices to mineral status have been obtained by methods which have evaluated bone development and density. Previous work has been concerned with perfecting techniques and instrumentation and in measuring bone density of subjects on self-selected or institutional diets; data to substantiate the reliability of these methods are meager. Bone density measurements began in this laboratory in 1948. McDonald ('49) reviewed some of the important work in the development of methods applicable to the assessment of calcium status. A review of the literature and an appraisal of the application of the photometric technique of measuring density of bones was given in Nutrition Reviews ('52).

The purpose of the present work was to study the effects on bone density of controlled changes in mineral content of the diet and to assess the bone index as a measure of calcium status. These studies were carried out over a period of 5 years and included 37 college students and more than 100 rats. The work in 1948 to 1949 involved measurements of bone density on a group of young adults who reported that their customary diets were low in milk. Comparable measurements were made after their usual diets had been supplemented with milk. Beginning in 1950 bone density values were obtained for young women serving as subjects for a metabolism experiment in which the diets supplied calcium

and phosphorus at low levels during periods of restriction and at or above recommended levels when supplemented with dicalcium phosphate or natural foods. Such a study afforded the opportunity to compare the bone densities of the same humans on their self-chosen diets and on controlled diets at both restricted and optimal mineral levels. In addition, comparative measurements were made on a series of rats, both young and mature, on diets of markedly different calcium content. Part of these rats were then used for radiocalcium studies to determine by this technique the effects of different calcium intakes on calcium reserves.

PROCEDURE FOR BONE DENSITY MEASUREMENTS

The advice of Mack and co-workers was helpful in obtaining the x-rays and in evaluating the results. The quantitative estimation of bone density from x-rays of the os calcis as developed by Mack has been described by Keys et al. ('50) and by Pennsylvania State College ('53). The procedure outlined in the latter publication is essentially that evolved in this laboratory since 1948. Blue Brand film was used for all the work reported in this paper; since 1950 the conditions for exposure for the x-ray of the heel bone and an ivory or alloy standard were 40 to 46 KV and 100 MAS with a focal-film distance of 36 in. Variation from this procedure in the preliminary work was in the use of a KV range of 36 to 52. The films were developed and mounted between two $4 \times 10 \times 0.040$ in. Eastman Kodak glass cover plates. From these films, tracings were made of a standard path across the x-ray image of both bone and ivory standard using a Leeds and Northrup recording microdensitometer. Mack's procedure was followed in obtaining a value for the density of bone plus flesh in terms of x-ray equivalent grams of ivory per unit volume of material. For the comparative purposes of this study, it was considered unnecessary to eliminate the x-ray absorption due to the over- and underlying flesh. The bone index so obtained is not an absolute

measure of the density of the bone but, in the text, the values will be referred to as the bone density.

The same general procedure was followed for the rat. The exposure conditions found to be satisfactory for x-raying the ivory standard and the vertebrae of the rat were 40 KV and 8 MAS at a distance of 40 in. The 9th caudal vertebra was selected for density analysis. On the assumption that both the tail and the vertebra through its center were essentially round the cross-sectional areas for flesh and bone were measured. Using the area and linear measurements derived from the vertebra traces, the density for the bone apart from the over- and under-lying flesh was calculated. This is recognized as an approximation rather than an absolute value. Analyses of data for reproducibility of measurements for bone density gave respective standard deviations of 0.18 and 0.14 for humans and rats.

EXPERIMENTAL AND RESULTS

Human studies

Series I. During 1948 to 1949, 13 students who indicated a low consumption of milk during their growing years kept a 7-day record of current food intake. X-rays of the heel bone were made for each subject and bone densities were calculated. Seven students who reported calcium intakes below the then recommended adult level of 1.0 gm per day were chosen for further study. One student reporting an intake of 1.1 gm per day was retained as a control. A 4-day balance study was conducted on a diet furnishing a calcium intake similar to that of the reported self-chosen diets. The calcium intake of all subjects was then brought to or above recommended levels by adding one, two or three cups of milk to the self-chosen diets. After three months on this milk-supplemented regime, x-rays were again taken and another balance study made. In 1949 to 1950, another group of 7 students volunteered for a similar experiment.

In table 1, the 20 subjects for the two years are arranged in order of decreasing calcium intakes as reported in their self-chosen diets. There was a significant decrease in bone density with decreased calcium intake as reported, $b = 0.33 \pm 0.08$. Six subjects (1, 6, 7, 10, 14 and 16) were eliminated from this analysis either because of evidence from the balance data that the self-chosen diets had been inaccurately estimated in relation to calcium intakes or because the subjects reported

TABLE 1

Calcium intake, calcium balance and bone index [1] of young adults

Series I

NO. OF SUBJECT	CALCIUM INTAKE			CALCIUM BALANCE		BONE INDEX [1]	
	Self-chosen diet (calculated)	Controlled diet (analyzed)	Supplemented diet (analyzed)	Controlled diet	Supplemented diet	Self-chose diet	Supplemented diet
	gm/24 hr.	*gm/24 hr.*	*gm/24 hr.*	*gm/24 hr.*	*gm/24 hr.*		
1	1.4	1.43	1.63	0.75	0.28	0.69	0.70
2	1.3					1.23	
3	1.2					1.15	
4	1.1	1.11	1.27	0.34	0.14	1.17	0.97
5 [2]	1.0					1.06	
6	1.0	1.12	1.35	0.53	0.05	0.81	0.74
7	1.0	1.12	1.35	0.60	0.31	0.88	0.74
8	0.9					1.08	
9	0.8	0.57	1.27	— 0.05	0.36	0.93	0.97
10	0.8	0.50	1.63	0.12	0.91	0.78	0.74
11	0.6					0.98	
12	0.6	0.57	1.12	— 0.01	0.22	1.06	0.94
13 [2]	0.6	0.59	1.41	— 0.14	0.19	1.06	1.07
14 [2]	0.6	0.59	1.41	— 0.05	0.27	1.21	1.12
15	0.5	0.57	1.12	— 0.07	0.05	0.90	0.94
16	0.5	0.57	0.87	0.10	— 0.11	1.14	1.24
17	0.5	0.50	1.06	0.26	0.03	0.86	0.88
18	0.4	0.57		— 0.01		1.08	0.96
19	0.3	0.50	1.06	0.17	— 0.06	0.64	0.68
20	0.2	0.50	1.35	0.28	0.43	0.92	0.97

[1] Number of x-ray equivalent grams of ivory per cubic centimeter of bone plus flesh.

[2] Male subjects.

that the diets were not typical of their habitual previous intakes. Following the three-month period of supplementation bone densities did not increase. These preliminary data, therefore, indicated that young adults had rather widely

TABLE 2

Average height, weight, age, calcium intake, calcium balance and bone index[1] of young adults

Series II

	1950–'51	1951–'52	1952–'53
Height (initial) — in.	64.6 ± 1.6	65.5 ± 1.5	66.0 ± 2.1
Weight (initial) — lb.	121[2] ± 16	123 ± 6	136 ± 8
Age (initial) — yr.	21.2 ± 1.3	20.8 ± 2.2	21.0 ± 1.5
Self-chosen diet			
Calcium intake gm/24 hr.	0.88	0.85	0.92
Bone index	0.93 ± 0.06	0.92 ± 0.05	0.84 ± 0.03
Restricted diet			
Calcium intake gm/24 hr.	0.32	0.36	0.26
Calcium balance gm/24 hr.	— 0.08 ± 0.03	— 0.13 ± 0.04	— 0.11 ± 0.03
Bone index	0.97 ± 0.04	0.92 ± 0.05	0.87 ± 0.04
Mineral-vitamin-supplemented diet			
Calcium intake gm/24 hr.	1.45	0.98	1.39
Calcium balance gm/24 hr.	0.07 ± 0.04	— 0.12 ± 0.04	0.00 ± 0.04
Bone index	0.91 ± 0.04	0.95 ± 0.04	0.89 ± 0.03
Milk-supplemented diet			
Calcium intake gm/24 hr.	1.46	1.02	1.29
Calcium balance gm/24 hr.	0.31 ± 0.04	0.12 ± 0.04	0.05 ± 0.04
Bone index	0.95 ± 0.08	0.92 ± 0.02	0.86 ± 0.04

[1] Number of x-ray equivalent grams of ivory per cubic centimeter of bone plus flesh.

[2] Excluding one obese subject who weighed 170 lbs.

varying values in respect to bone density which appeared to be related to previous long-continued dietary habits and were not readily affected by marked temporary changes in calcium intake.

Series II. For these experiments the subjects were college women between the ages of 19 and 25 years. The experimental

regime for these subjects on the restricted, the mineral-vitamin- and the milk-supplemented diets has been described elsewhere (Schofield et al., '56). X-rays were made initially and after each experimental period. Average bone density measurements are presented in table 2.

Records of self-chosen diets indicated that about 70% of the subjects were receiving at least 0.8 gm of calcium per day. Of the 30% receiving less than 0.8 gm, all were obtaining enough calcium to meet the average adult maintenance requirement of 6.4 mg per kilogram, as reported by Sherman ('20), and only one reported less than the 10 mg per kilogram, as suggested by Steggerda and Mitchell ('41).

When the subjects were placed on a diet with a daily calcium content of 0.3 to 0.4 gm, the average balances for all subjects were negative. This indicated that the self-chosen diets had actually contained more calcium than the restricted ones. When a calcium supplement in the form of a mineral was added to the diet to give a total of 1.0 gm, one of the subjects changed from a negative balance to equilibrium while the others remained in negative balance. When the mineral was added to bring the total daily calcium to 1.4 gm, on the average, the shorter, lighter subjects (1950–'51) stored calcium and the taller, heavier subjects (1952–'53) achieved equilibrium only, the two tallest and heaviest subjects remaining in negative balance. When 1.0 to 1.5 gm of calcium was furnished by natural foods, all the subjects either stored calcium or came to equilibrium.

No significant differences in average bone densities occurred as a result of temporary changes in calcium intake under the conditions of this experiment. Mean bone densities for all subjects on self-chosen, restricted, mineral-vitamin-supplemented and milk-supplemented diets were 0.89, 0.91, 0.91 and 0.91 respectively. It will be noted, however, that the average bone densities for the 1952 to 1953 subjects were consistently and significantly lower than for those in the other two years. The larger women apparently had a higher requirement for calcium than those who were smaller. These data confirm the

preliminary study indicating that bone densities of normal young women vary and are not readily altered by marked temporary changes in calcium intake. The metabolism and bone density studies on young adult humans indicated that calcium balance tends to reflect the immediately previous calcium intake and the availability of the mineral while bone density represents the cumulative effects of over-all past nutritional history.

Rat studies

Because of the indications obtained in the human studies that bone density measurements are not influenced by temporary reduction or increase in the calcium intake of young adults, two questions were raised: whether similar marked changes in the calcium intake of adults would result in alteration of bone density if the dietary change were continued over longer periods; and whether such changes in calcium intake might more readily influence bone density of younger individuals during periods of rapid growth. The use of rats seemed to offer a means of obtaining at least partial answers to these questions.

Young rats. Four groups of 11 rats each were assigned to diets containing 0.1, 0.3 and 0.5% of calcium. The animals were approximately 4 weeks old and the average weight of those in each group was 53 to 54 gm. The dietary phosphorus was maintained at 0.4% because it seemed more important to keep the diets adequate in all nutrients except calcium than to maintain a constant calcium to phosphorus ratio. Littermates were fed these diets in equal amounts for 6 to 7 weeks and a 4th littermate was given ad libitum the diet containing the highest level of calcium.

At the beginning and end of the experiment the animals were lightly anesthetized and x-rays of the 9th caudal vertebra were taken for bone density measurements. After the final x-rays the rats were sacrificed, the bodies ashed and the ash analyzed for calcium. Analysis of the data (table 3) shows

that at the dietary levels studied the calcium content of the rat was dependent upon intake, the body calcium increasing at a constant rate as dietary calcium was increased, $b = 0.64 \pm 0.04$. Similarly, final bone density was directly related to both calcium intake, $b = 0.23 \pm 0.03$, and calcium content of the rat, $b = 0.33 \pm 0.04$. A small but significant deviation from linearity of bone density response to intake ($P < 0.05$) indicated that the maximum bone density was being approached at the highest level of restricted

TABLE 3

Weight gain, calcium content and bone index [1] of rats fed at different levels of calcium intake

DIETARY CALCIUM	NO. OF RATS	CALCIUM INTAKE	GAIN IN WEIGHT	CALCIUM CONTENT	BONE [1] INDEX
%		*mg/rat/day*	*gm*	*gm./rat*	
Young rats					
0.1	11	7.2	75 ± 7	0.60 ± 0.05	0.39 ± 0.02
0.3	11	21.7	79 ± 6	1.08 ± 0.05	0.60 ± 0.04
0.5	11	31.8	80 ± 6	1.25 ± 0.06	0.67 ± 0.02
0.5 ad lib	11	40.3	125 ± 7	1.48 ± 0.06	0.71 ± 0.04
Mature rats					
0.1	8	9.5	64 ± 13	3.02 ± 0.21	0.78 ± 0.05
0.3	8	28.2	61 ± 8	3.28 ± 0.26	0.90 ± 0.05
0.5	8	43.4	71 ± 11	3.20 ± 0.28	0.82 ± 0.04
0.5 ad lib	8	44.6	101 ± 11	3.46 ± 0.34	0.85 ± 0.06

[1] Number of x-ray equivalent grams of ivory per cubic centimeter of bone.

calcium intake. Increasing the total intake by ad libitum feeding of the diet containing 0.5% of calcium also increased the rate of deposition ($P < 0.01$). Therefore, maximum bone density apparently had not been reached when the animals ate the diet containing 0.5% of calcium in somewhat restricted amounts. When isocaloric amounts of food were eaten, there was no tendency for greater gains in weight at the higher calcium intakes; however, rats fed the 0.5% calcium diet ad libitum grew more rapidly ($P < 0.01$).

Mature rats. A littermate series with 8 adult rats in each group was put on diets of 0.1, 0.3 and 0.5% calcium content, respectively, with equalized food consumptions for a period of approximately 9 months. A 4th group of littermates was given the 0.5% calcium diet ad libitum. The rats initially were between three and 4 months in age and had been raised on a stock diet containing 0.3% of calcium. The average initial weights for each group were 210, 214, 215 and 210 gm, respectively.

On isocaloric food intakes, slightly more calcium was stored on the two higher calcium intakes than on the low. When the 0.5% calcium diet was fed ad libitum there was a still further increase in total calcium stored. The regression of body calcium on intake indicates that the increased storage is small but significant since $b = 0.08 \pm 0.03$.

No significant difference in bone densities of mature rats was found after 9 months on different levels of calcium. Thus over this period which represented a long time in the life span of the rat, evidence was lacking to indicate that bone densities were affected by the different levels of calcium fed. A similar situation occurred for humans on fairly comparable levels for much shorter durations. The average bone index of rats on the lowest calcium intake, however, was slightly lower than that of those on the higher calcium levels.

When isocaloric amounts of food were eaten there was no tendency for greater gains in weight at the higher calcium intakes. Mature rats fed the 0.5% calcium diet ad libitum grew more than those restricted in diet ($P < 0.01$) but at a much slower rate (2.8 gm/wk.) than did the corresponding young animals (20.8 gm/wk.).

Radiocalcium studies.[1] After the final x-rays, 5 young and two mature animals from each group were given an oral dose of Ca^{45}. The animals were kept in metabolism cages for

[1] This work was done by one of the authors (B. B. M.) at UT — AEC Laboratory while an Oak Ridge Institute of Nuclear Studies Fellow on leave from the University of Tennessee.

a 72-hour balance study and then sacrificed. The balance data are summarized in table 4.

The fecal excretion figures indicate that more stable calcium was eliminated by both young and mature animals with higher dietary calcium. At the same level of intake the young growing rats excreted less than the mature ones. When radiocalcium was given, the percentage of the Ca^{45} dose which was excreted increased (or the percentage absorbed decreased) with higher dietary calcium for the rats which had been fed isocaloric amounts of food. The percentage of the Ca^{45} dose excreted did not increase with ad libitum feeding which supplied still more dietary calcium and resulted in added growth.

TABLE 4

Calcium[45] retention in young and mature rats as a function of the plane of calcium nutrition

CALCIUM IN DIET	CALCIUM INTAKE	FECAL CALCIUM	FATE OF Ca^{45} DOSE		ENDOGENOUS FECAL CALCIUM	UNABSORBED CALCIUM
			Feces	Retained		
%	*mg/rat/day*		%	%	*mg/rat/day*	
Young rats						
0.1	2.9	1.0	0.2	99.8	1.0	0
0.3	9.7	1.5	3.5	96.5	1.4	0.1
0.5	15.1	6.0	15.7	84.3	3.9	2.1
0.5 ad lib	29.5	12.3	15.5	84.5	8.2	4.1
Mature rats						
0.1	2.3	3.2	8.5	91.5	3.0	0.2
0.3	7.2	12.4	13.1	86.9	11.6	0.8
0.5	20.5	29.6	26.0	74.0	24.5	5.1
0.5 ad lib	43.3	34.1	19.0	81.0	25.9	8.2

The use of radiocalcium made possible the partition of total excretion into unabsorbed and endogenous calcium. By using the formula derived from the comparative balance method described by Comar et al. ('53) for cattle and modified for rats by Hansard and Plumlee ('54) the amount of endogenous calcium was estimated. The endogenous fecal calcium was greater at increased intakes for both the young and mature animals, with the older animals showing the larger amounts at a given calcium level. Since, for the

young animals at the two lower levels of intake, the endogenous accounted for nearly all the total excretion, practically no calcium was unabsorbed. While percentage absorption decreased, total absorption and retention of calcium were greater with increased intake, and the calculations for the two higher levels gave increasingly larger amounts for both endogenous and unabsorbed calcium. It would thus seem that as body stores were better filled and growth needs adequately met by higher calcium intakes over a period of time, the animals responded by decreased percentage absorption and by a greater absolute turnover of calcium already present in the bones as indicated by the increased endogenous excretion.

Specific activities of the carcasses and of the vertebrae of the rats are presented in table 5. Comparable figures were obtained for the tibia shaft and epiphysis. When isocaloric amounts of food were fed to the animals at the different calcium levels, growth was not significantly different (table 3). A direct comparison of specific activities can therefore be made without the complications due to differences in growth. For young rats specific activity decreased ($P < 0.01$) with increased calcium intake. In the mature rats the same trend was evident in the few animals studied. When ad libitum feeding was allowed for the young animals, the additional calcium and other nutrients supported a significant gain in weight and a resultant greater, but not proportionate, storage of calcium. Subsequently these animals exhibited specific acitvities as great as those on the same diet restricted in amount. In other words, when the animals were allowed to eat freely at the high calcium level the downward trend of calcium activity was reversed to meet the needs for the additional growth (7.5 gm/wk.). In mature animals that had been started on the experiment after three to 4 months of adequate nutrition, the calcium reserves were so nearly filled at all levels of intake, as indicated by the relatively small amount of activity shown by all animals, that the small added growth (0.8 gm/wk.) allowed by the ad libitum

TABLE 5

Distribution of radiocalcium in young and mature rats maintained at different calcium levels

CALCIUM IN DIET	NO. RATS	CARCASS [1]				VERTEBRA		
		Total Ca		Ca^{45} dose [2]/gm	S.A.[3]	Total Ca	Ca^{45} dose [2]/gm	S.A.[3]
%		*mg/rat*	*mg/gm*	%		*mg/gm*	%	
Young rats								
0.1	5	565	5.12 ± 1.59	0.775 ± .076	0.161	18.8	1.81	0.099
0.3	5	1004	8.28 ± 1.82	0.789 ± .057	0.103	32.8	2.36	0.077
0.5	5	1115	8.88 ± 0.83	0.706 ± .065	0.096	47.0	2.34	0.059
0.5 ad lib	5	1414	7.83 ± 0.60	0.667 ± .105	0.101	51.0	2.78	0.072
Mature rats								
0.1	2	3280	11.0 ± 1.2	0.634 ± .040	0.064	55.1	1.56	0.028
0.3	2	3665	11.8 ± 0.4	0.566 ± .059	0.056	48.4	1.39	0.028
0.5	2	3270	10.2 ± 1.1	0.384 ± .054	0.050	47.9	1.02	0.021
0.5 ad lib	2	3978	12.0 ± 0.9	0.332 ± .052	0.034	56.0	0.91	0.016

[1] Carcass consisted of whole body minus gastrointestinal tract and portions of bone removed for analysis.

[2] Corrected to 100 gm body weight.

[3] Specific activity = % dose (based on *retained* dose) per mg Ca.

feeding was not enough to check the downward trend in activity.

Specific activities gave confirmation of the results obtained from chemical analyses and bone density measurements that the calcium reserves of young rats increased with increased calcium intakes. They also suggested that in older animals a similar increase in calcium reserves occurred with increased intake. The differences in calcium content of the latter were too small to be detected by the bone density measurements.

DISCUSSION

In the growing rat bone density measurements from x-ray photographs were adequate to detect the influence of different levels of dietary calcium; in the mature rat and the young human no changes were observed. Absence of demonstrable change in the adult is in disagreement with the findings of Mack [2] that a radical change in the level of dietary calcium produces a measurable change in bone density of adults within a few days. Bone density values were in agreement with the results of chemical analyses in rats but not with balance data for the human subjects in the metabolism experiment. Bone density appears to be a measure of calcium status determined by the past nutritional and developmental history while balance reflects the immediately previous calcium intake. Steggerda and Mitchell ('46) warn against using requirements arrived at from balance experiments in forming decisions regarding the prevalence of calcium undernutrition. They emphasize the great variability shown by the human organism in its disposal of dietary calcium and its marked ability to adapt to wide ranges in calcium intake. That humans adapt to low calcium intakes has been shown by Nicholls and Nimalasuriya ('39), Basu et al. ('39), Owen and Irving ('40) and Hegsted et al. ('52). Steggerda and Mitchell ('41), in discussing some of these studies which showed that calcium equilibrium is attainable at levels from 168 to 280 mg per day,

[2] Personal communication. Dr. P. B. Mack, Texas State College for Women.

stated that some of the subjects who were beyond middle life showed evidences of osteoporosis and that possibly all cases, even after successful adjustment to the low intake of calcium, were not in the best of health.

Henry and Kon ('53) demonstrated that rats adapt to high as well as to low dietary calcium. Their animals maintained on a high calcium diet grew more and had a higher calcium requirement than rats which had been on a low calcium regime. When these animals were placed on a lower calcium diet the balances became negative whereas the rats maintained on the low calcium diet continued in equilibrium. It should not be said that the low calcium intake was desirable simply because the animals were in equilibrium unless it can be said also that the retarded growth and lowered calcium reserves were physiologically desirable. Proof for this latter statement is lacking whereas there is some evidence to the contrary. When rats were fed diets higher in calcium than usually considered adequate, they achieved earlier attainment of maturity, a longer period of reproductive activity and postponement of the onset of old age (Sherman and Campbell, '35; Campbell et al., '43).

Steggerda and Mitchell ('46) kept one human subject on low calcium intakes for as long as 192 days. At the end of this time the average calcium balance for the whole period was still negative. The Tennessee studies with adults, while indicating no differences in bone density at different calcium intakes for either rat or human, gave some evidence in rats for small but significant differences in body calcium and in calcium reserves as shown by the radiocalcium studies. If even small losses continue to occur at low calcium intakes, which may be habitual in older people, these losses might explain, in part at least, the incidence of senile osteoporosis which is known to exist. Both Hegsted et al. ('52) and Steggerda and Mitchell ('41) point out that further studies are needed in which objective evidence of malnutrition must be the criterion used in seeking a correlation with dietary calcium deficiency. Vavich et al. ('54) and Mack and associates

('39, '42, '47, '49a,b) have reported that in growing children bone density values show significant changes with alteration in the quality of the diet. The studies reported here gave evidence that in adults also bone density measurements may offer a method for establishing norms of calcium status.

SUMMARY

1. Bone densities of young human adults showed variations which appeared to indicate the calcium status of the individual.

2. Characteristic bone densities in young adults were not readily altered by marked temporary changes in calcium intake whereas calcium balances readily reflected such differences.

3. Mature rats showed no significant changes in bone density but a small and significant difference in calcium content with increasing intakes ranging from 0.1 to 0.5% of calcium.

4. Young growing rats showed significant increases both in bone density and body calcium with increased intake ranging from 0.1 to 0.5% of calcium.

5. Specific activities decreased with increased calcium intakes for mature rats and for growing rats on controlled food intakes. The decrease in activity was interpreted as an indication of increase in calcium reserves.

6. The implications of the use of bone density measurements for determining calcium status have been discussed.

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LITERATURE CITED

BASU, K. P., M. N. BASAK AND B. C. RAI SIRCAR 1939 Studies in human metabolism. Part II. Calcium and phosphorus metabolism in Indians on rice and on wheat diets. Ind. J. Med. Res., *27:* 471–499.

CAMPBELL, H. L., C. S. PEARSON AND H. C. SHERMAN 1943 Effect of increasing calcium content of diet upon rate of growth and length of life of unmated females. J. Nutrition, *26:* 323–325.

COMAR, C. L., R. A. MONROE, W. J. VISEK AND S. L. HANSARD 1953 Comparison of two isotope methods for determination of endogenous fecal calcium. Ibid., *50:* 459–467.

HANSARD, S. L., AND M. P. PLUMLEE 1954 Effects of dietary calcium and phosphorus levels upon the physiological behavior of calcium and phosphorus in the rat. Ibid., *54:* 17–31.

HEGSTED, D. M., I. MOSCOSO AND C. COLLAZOS 1952 A study of the minimum calcium requirements of adult men. Ibid., *46:* 181–201.

HENRY, K. M., AND S. K. KON 1953 The relationship between calcium retention and body stores of calcium in the rat: effect of age and vitamin D. Brit. J. Nutrition, *7:* 147–159.

KEYS, A., J. BROZEK, A. HENSCHEL, O. MICKELSEN AND H. L. TAYLOR 1950 The biology of human starvation. University of Minnesota Press, Minneapolis, Vol. II.

MACK, P. B. 1949a Comparison of meat and legumes in a controlled feeding program. IV. Second period with regimens reversed — dietaries and results of physical observations. J. Am. Diet. Assn., *25:* 848–857.

———— 1949b Comparison of meat and legumes in a controlled feeding program. VI. Seven-month study of twenty-four children. Ibid., *25:* 1017-1021.

MACK, P. B., V. D. SHEVOCK AND M. R. TOMASSETTI 1947 Comparison of meat and legumes in a controlled feeding program. III. Discussion of findings. Ibid., *23:* 677–685.

MACK, P. B., AND J. M. SMITH 1939 Methods of conducting mass studies in human nutrition. The Pennsylvania State Col. Bull. The Pennsylvania State College Studies, Study No. 4.

MACK, P. B., J. M. SMITH, C. H. LOGAN AND A. T. O'BRIEN 1942 Mass studies in human nutrition: Nutritional status of children in a college community. J. Am. Diet. Assn., *18:* 69–78.

MCDONALD, B. B. 1949 Bone density measurements as related to dietary calcium in humans. M. S. thesis problem. University of Tennessee.

NICHOLLS, L., AND A. NIMALASURIYA 1939 Adaptation to a low calcium intake in reference to the calcium requirements of a tropical population. J. Nutrition, *18:* 563–577.

NUTRITION REVIEWS 1952 Bone density. *10:* 119–124.

OWEN, E. C., AND J. T. IRVING 1940 The calcium requirements of older male subjects with special reference to the genesis of senile osteoporosis. Acta Med. Scand., *103:* 235–250.

PENNSYLVANIA STATE COLLEGE 1953 Instructions for exposing and developing x-ray films for bone density evaluation. 3rd rev. ed. State College, Pennsylvania.

SCHOFIELD, F. A., D. E. WILLIAMS, E. MORRELL, B. B. MCDONALD, E. BROWN AND F. L. MACLEOD 1956 Utilization of calcium, phosphorus, riboflavin and nitrogen on restricted and supplemented diets. J. Nutrition, *59:* 561–577.

SHERMAN, H. C. 1920 Calcium requirement of maintenance in man. J. Biol. Chem., *44:* 21–27.

SHERMAN, H. C., AND H. L. CAMPBELL 1935 Effects of increasing the calcium content of a diet in which calcium is one of the limiting factors. J. Nutrition, *10:* 363–371.

STEGGERDA, F R., AND H. H. MITCHELL 1941 Further experiments on the calcium requirement of adult man and the utilization of the calcium in milk. Ibid., *21:* 577–588.

———— 1946 Variability in the calcium metabolism and calcium requirements of adult human subjects. Ibid., *31:* 407–422.

VAVICH, M. G., A. R. KEMMERER AND J. S. HIRSCH 1954 The nutritional status of Papago Indian children. Ibid., *54:* 121–132.

EFFECT OF INTAKE LEVEL ON THE UTILIZATION AND INTESTINAL EXCRETION OF CALCIUM IN MAN [1]

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The absorption of calcium contained in various foods cannot be directly determined by metabolic balances as the difference between dietary calcium intake and fecal calcium loss. The stool contains calcium secreted with the digestive juices into the gastrointestinal tract (endogenous fecal calcium) in addition to the calcium of the food which passes unabsorbed. It is now possible to differentiate these two stool components by the isotope dilution technique. This technique was first used in man for the estimation of endogenous fecal phosphorus by Hevesy et al. ('39) and for the estimation of endogenous fecal calcium in cattle by Visek et al. ('53). The endogenous fecal calcium and the fraction of dietary calcium which passes unabsorbed was measured in man in this laboratory by tagging the body calcium with radiocalcium. The utilization of calcium was measured by this technique in two patients maintained on a low calcium diet; these results were previously reported (Blau et al., '54). This communication extends these studies to the effects of varying calcium intake levels.

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Measurement of endogenous fecal calcium and utilization of dietary calcium. When a tracer dose of radioactive calcium is administered intravenously or orally, equilibration of this tracer with the exchangeable portion of the body calcium takes place and the specific activities of the plasma, urine and digestive juices are the same (Visek et al., '53; Blau et al., '54). Following the excretion of the unabsorbed portion of an oral dose, and at all times after an intravenous dose, the calcium of the stool has a lower specific activity than the body fluids because the specific activity of the digestiye juices is diluted with non-radioactive unabsorbed food calcium. Therefore

$$\text{E.F.C.} = \frac{\text{S.A. stool}}{\text{S.A. end}} \times \text{total stool calcium}$$

where S.A. stool = specific activity of stool, S.A. end. = specific activity of endogenous calcium, i.e., urine or plasma, and E.F.C. — endogenous fecal calcium.

Having determined the endogenous fecal calcium the unabsorbed food calcium can be calculated as follows:

Unabsorbed food calcium = Stool calcium — E.F.C. and, therefore, the absorption or utilization of dietary calcium can be measured by:

$$\text{Utilization \%} = \frac{\text{Intake} - \text{Unabsorbed food calcium}}{\text{Intake}} \times 100$$

The validity of the above calculations depends on the assumption that the body fluids contain calcium of uniform specific activity, i.e., that the body does not discriminate between radioactive and stable calcium in the physiologic processes of filtration and secretion. The good agreement of the specific activties of plasma and urine has been previously demonstrated (Blau et al., '54).

Estimation of the total digestive juice calcium. It is assumed that large amounts of calcium are secreted with the digestive juices into the intestinal tract. The preceding section outlined the method for the determination of endogenous

fecal calcium, i.e., the fraction of the digestive juice calcium which has not been re-absorbed. Can the total digestive juice calcium also be measured?

Let us assume that the ingested calcium mixes homogeneously and exchanges with the calcium of the digestive juices and that absorption takes place subsequently from this intestinal pool. The stool is the unabsorbed residue of this mixture and has the same specific activity as the pool. The radioactive calcium of this pool equals the total digestive juice calcium multiplied by its specific activity (which equals that of plasma or urine); the total calcium in the intestinal pool is the sum of the ingested and the digestive juice calcium. Therefore,

$$\text{S.A. stool} = \frac{\text{Radioactive calcium}}{\text{Total calcium}} = \frac{\text{T.D.J. Ca} \times \text{S.A. end}}{\text{T.D.J. Ca} + \text{Ingested Ca}}$$

or

$$\text{T.D.J. Ca} = \text{Ingested Ca} \times \frac{\text{S.A. stool}}{\text{S.A. end} - \text{S.A. stool}}$$

where T.D.J. Ca = total digestive juice calcium, S.A. stool = specific activity of stool, S.A. end = specific activity of endogenous calcium.

The value for the total digestive juice calcium obtained from these calculations represents a maximum value. Two assumptions had to be made: (1) Complete mixing of food and intestinal juices before absorption proceeds and (2) Once mixing has occurred the absorption process does not distinguish between food calcium and endogenous calcium. It is unlikely that complete mixing of dietary calcium and intestinal juice calcium takes place *before* absorption. For instance, radiocalcium can be detected in the blood 15 to 30 minutes after the ingestion of the tracer, indicating its partial absorption in the upper intestinal tract prior to mixing with the digestive juices secreted into the lower intestine (Blau et al., '54). Absorption of food calcium before mixing would lead to a higher specific activity and therefore to a spuriously high value for the total digestive juice calcium. Since it is

probable that absorption occurs in part before mixing, the values for the total digestive juice calcium calculated by this method are maximal. It is unlikely that the body can distinguish between endogenous and exogenous calcium during the process of absorption. Although the chemical form of calcium contained in the food is different from that of the digestive juices, mixing and exchange can be assumed to take place between the radioactively-labelled digestive juice calcium and even such insoluble compounds as calcium phosphate or phytate unless the food calcium is mechanically inaccessible for mixing. This latter possibility is remote under physiologic conditions. Exchange of ingested Ca^{++} with endogenous Ca^{++} across the intestinal wall may also lead to high values for digestive juice calcium. It is assumed that the maximal values obtained for the digestive juice calcium are close approximations of the true values in physiologic conditions of the gastrointestinal tract. Whether this is also true in pathologic states of the gastrointestinal tract will need careful evaluation in each case.

SUBJECTS AND METHODS

Two fully ambulatory patients, I.B. and B.L., were studied for 74 days on the metabolic research ward.

Patient I.B., a 52 year old white female, body height 156 cm, body weight was 50.2 kg., had two conditions: (a) giant follicular lymphoblastoma, diagnosed 7 years ago; ionizing radiation was given to several portals. However, no therapy was given in the three years prior to the study since the condition was quiescent. (b) Carcinoma of the uterus treated with radiation and hysterectomy. The metabolic study was started 5 weeks after the latter. Temperature, respiratory rate, blood pressure, complete blood count, blood proteins and renal functions were normal. Serum calcium was 10.3 mg%, serum phosphorus 3.8 mg%, alkaline phosphatase 3.2 Bodansky units.

Patient B.L., a 72 year old male, body height 163 cm, body weight 67.5 kg., had a total laryngectomy performed for squamous cell carcinoma of the larynx 4 years prior to the

study. He had a tracheotomy; there was no evidence of local recurrence or distant metastases on clinical or laboratory examination at the time of the study or even two years after completion of the study. Temperature, respiratory rate, blood pressure, complete blood count, blood proteins and renal functions were normal. Serum calcium was 10.0 mg%, serum phosphorus 2.8 mg%, alkaline phosphatase 4.0 Bodansky units.

Both patients were placed on a low-calcium diet (Bauer and Aub, '27) which was maintained throughout the entire study. The diet of patient I.B. contained 1540 calories (51.3 gm protein, 209 gm carbohydrates and 49.8 gm fat); the diet of patient B.L. contained 1800 calories (64.1 gm protein, 284 gm carbohydrates and 62.2 gm fat). Fluid intake and output, and the body weights of the patients were determined daily. No significant fluctuations of body weight were noted in either patient during the study. The average dietary nitrogen, calcium and phosphorus intake and the balances are listed in table 1. The study was divided into three phases: in the first four 6-day metabolic periods, the patients received no calcium gluconate supplementation (periods 1 to 4); in the subsequent four 6-day metabolic periods I.B. received 422 mg and B.L. 503 mg of calcium per day in the form of calcium gluconate tablets given with meals (periods 5 to 8); in the last four 6-day metabolic periods, the calcium supplement was raised to 1339 mg/day in I.B. and to 1605 mg/day in B.L. (periods 9 to 12). The Ca:P ratio was 1:5 in the first phase, 1:1 in the second phase and 2.6:1 in the third phase of the study. Two days prior to the first metabolic period each patient received with breakfast a single oral tracer dose of 50 μc of Ca^{45} to which 30 mg of calcium carrier were added.

The nitrogen, calcium and phosphorus content of the food was determined on aliquots of the diet of each metabolic period. The urinary creatinine was determined daily. The urinary and fecal nitrogen were determined by the Kjeldahl method, the phosphorus by the method of Fiske and Subbarow ('25) on dried, ashed aliquots of 6-day metabolic pool collections. The urinary and fecal calcium were measured

TABLE 1

Metabolic balances

PERIODS [1]	CALCIUM, *mg/24 hours*				NITROGEN, *mg/24 hours*				PHOSPHORUS, *mg/24 hours*			
	Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance
PATIENT I.B.												
1–4	107	90	151	— 134	8990	5120	910	+ 2960	534	269	153	+ 112
5–8	529	85	408	+ 36	9150	6550	1040	+ 1560	511	259	182	+ 70
9–12	1446	103	733	+ 610	9160	6490	1220	+ 1450	546	270	229	+ 43
PATIENT B.L.												
1–4	135	51	162	— 78	11250	8150	1100	+ 2000	676	366	225	+ 85
5–8	638	66	278	+ 294	11300	8710	750	+ 1840	632	235	298	+ 99
9–12	1740	92	774	+ 874	11450	8910	1250	+ 1290	682	250	367	+ 65

[1] Each metabolic period consists of 6 days. Each value is an average of 24 days.

daily during periods 1 to 4, and on aliquots of 6-day pool collections thereafter. The daily urinary calcium was determined by the method of Shohl and Pedley ('22); the calcium content of stool and of the urine pools was determined on dried, acid-ashed samples. The serum calcium was determined at frequent intervals in the early phase of the study and once weekly thereafter by the method of Kramer and Tisdall ('21).

The radioactivity was determined in duplicate on 1- to 5-ml samples of each 24-hour urine collection and on each stool specimen for the first 26 days of the study; thereafter it was determined on aliquots of urine and of stool pools. The radioactivity of the plasma was determined on 1- to 2-ml serum samples. The Ca^{45} level of the plasma became too low to be reliably measured by the end of the 4th metabolic period.

For the radioactivity determinations, 15 mg of Ca^{++} carrier were added to each sample followed by 5 ml of saturated ammonium oxalate. Acidity was adjusted to about pH 4 with ammonium acetate-acetic acid buffer (just acid to methyl orange). After standing over night the bulk of the supernatant liquid was decanted and a few milligrams of colloidal graphite [4] were added to the suspension to render the final sample conducting, preventing spurious counting due to the building of surface charge in the windowless counter. The suspension was transferred to a centrifuge device which deposited the calcium oxalate evenly on $1\frac{1}{8}'' \times \frac{1}{8}''$ planchets. The excess water was aspirated off and the samples were air dried at room temperature for 16 to 24 hours. To avoid dusting of the sample in the counter, 0.2 ml of a 2% Saran solution in methylethylketone was carefully pipetted on to each planchet. After final air drying for another 4 hours duplicate counts were taken on each sample in a windowless flow-gas Geiger counter. Samples were counted for a minimum of 2000 counts giving a counting statistics error of

[4] Aquadag, Acheson Colloids Co., Port Huron, Michigan.

about 2%. All counts were compared to standards prepared in an identical manner from aliquots of the administered dose of Ca^{45}.

Since the standards were always recounted together with any group of samples it was unnecessary to correct counts for decay or changes in counter efficiency. All counts were corrected for self-absorption using an experimentally determined correction curve. The radioactivity of the samples is expressed as the percentage of the administered dose.

RESULTS AND DISCUSSION

Metabolic balances (table 1). The calcium balances of both patients were slightly negative on a low-calcium intake, became slightly positive on the intermediate and markedly positive on the high-calcium intake, with retentions of 610 and 874 mg/day respectively. In spite of the marked increment of calcium intake, the urinary calcium excretion rose only slightly, the highest increment was 41 mg calcium/day in patient B.L. This observation is in accord with our previous experience and with data of Nicolaysen et al. ('53) and Laszlo et al. ('52). The serum calcium levels remained essentially unchanged during the entire study and ranged from 10.0 to 10.5 mg % in I.B. and from 9.5 to 10.4 mg % in B.L.

The phosphorus balances showed only slight fluctuations during the entire study. A decrease in urinary phosphorus and an increase in fecal phosphorus was noted with increasing calcium intake in B.L., the fecal phosphorus of I.B. also increased slightly while the urinary phosphorus remained essentially unchanged.

Theoretical phosphorus balances were calcaulated for both patients, assuming a Ca : P ratio of 2.2 : 1 and N : P ratio of 14.7 : 1. While good agreement was noted in both patients between the actual and theoretical phosphorus balances on the low-calcium intake, increasing discrepancies were observed on the higher levels of calcium intake, less phosphorus

was retained than the theoretical Ca: P ratio would require. This observation confirms our previous data (Laszlo et al., '52) and seems to indicate that the retained calcium was not deposited in bone in the form of phosphate or that phosphorus was shifted from other body depots to bone.

The nitrogen balances were positive in both patients throughout the entire study.

Ca^{45} metabolism (table 2). The Ca^{45} metabolism of the two patients during the first 26 days of low-calcium intake was previously reported (Blau et al., '54). Some of these data and those for the intermediate and high levels of calcium intake are listed in table 2. The specific activity of the urine continued to fall smoothly throughout the entire experiment while the specific activity of the stool declined sharply with each increase of calcium intake.

Patient I.B. excreted in the stool 64% of the ingested dose in 74 days; of this, 57% was the unabsorbed dose and 7% was calculated to be endogenous; 43% of the dose was absorbed. Expressing the endogenous fraction in percentage of the *absorbed* dose, this fraction is 16%. The cumulative urinary excretion was 8.2% of the ingested dose or 19% of the absorbed dose. The corresponding values for subject B.L. are: 45% of the dose was excreted in the stool in 74 days; of this 33% was the unabsorbed dose and 12% was endogenous, 67% was absorbed. The endogenous fecal fraction of 12% corresponds to 18% of the absorbed dose. The cumulative urinary excretion was 5.7% of the administered dose or 8.5% of the absorbed dose.

Endogenous fecal calcium (table 3). The values for the endogenous fecal calcium were calculated for both patients on the three levels of intake as described above. In spite of the major changes in the dietary intake levels no significant changes occurred in the excretion of endogenous fecal calcium. It can also be noted that the endogenous fecal calcium losses are of a similar order of magnitude as the urinary calcium excretion (table 1).

TABLE 2

Calcium and radiocalcium excretion

PERIODS [1]		URINE				STOOL			
		Ca^{45}	Ca	Specific activity	Ca^{45} cum. exc.	Ca^{45}	Ca	Specific activity	Ca^{45} cum. exc.
		% dose/day	*mg/day*	*% dose/100 mg Ca*	*% dose*	*% dose/day*	*mg/day*	*% dose/100 mg Ca*	*% dose*
		PATIENT I.B.							
Low	1	0.524	88	....	4.19	....	130	....	59.2
	2	0.201	81	0.249	5.39	0.287	173	0.166	60.9
	3	0.140	90	0.156	6.23	0.191	195	0.098	62.0
	4	0.086	103	0.085	6.74	0.080	123	0.066	62.5
Intermediate	5	0.065	99	0.066	7.17	0.076	338	0.023	63.3
	6	0.051	77	0.066	7.48	0.053	520	0.010	63.6
	7	0.032	80	0.040	7.67	0.014	167	0.0084	63.7
	8	0.022	86	0.026	7.80	0.028	604	0.0046	63.9
High	9	0.023	94	0.024	7.94	0.014	730	0.0019	64.0
	10	0.015	91	0.017	8.03	0.012	770	0.0015	64.1
	11	0.015	126	0.012	8.12	0.0069	592	0.0012	64.1
	12	0.012	102	0.012	8.19	0.0094	842	0.0011	64.2
		PATIENT B.L.							
Low	1	0.319	53	....	2.55	....	137	....	38.3
	2	0.117	48	0.244	3.26	0.203	90	0.228	39.5
	3	0.076	48	0.158	3.71	0.242	180	0.135	41.0
	4	0.062	53	0.117	4.08	0.160	217	0.074	41.9
Intermediate	5	0.056	65	0.086	4.40	0.033	114	0.029	42.8
	6	0.041	67	0.061	4.65	0.088	342	0.026	43.3
	7	0.039	64	0.061	4.88	0.065	403	0.016	43.7
	8	0.039	68	0.056	5.11	0.033	255	0.013	43.9
High	9	0.031	71	0.044	5.30	0.045	745	0.0061	44.2
	10	0.023	91	0.025	5.43	0.019	672	0.0028	44.3
	11	0.025	98	0.025	5.58	0.011	520	0.0021	44.4
	12	0.022	108	0.020	5.72	0.028	1160	0.0024	44.6

[1] Each metabolic period consists of 6 days.

Total digestive juice calcium (table 3). The average total digestive juice calcium (T.D.J.Ca) estimated for I.B. was 150 mg/day, for B.L. 290 mg/day. High calcium intake had no significant effect on the T.D.J. Ca in I.B. while it decreased from 360 to 240 mg/day in B.L. As discussed previously, the values for T.D.J. Ca are maximal although they are probably close approximations to the true values in these two patients with a normally functioning gastrointestinal tract. The figures for T.D.J. Ca are lower than those cited by Nicolaysen et al. ('53) on the basis of estimations in the literature. Figures derived from actual determinations of the total digestive juice calcium are not available for comparison.

Utilization of ingested calcium (table 3). The data presented in this study permit the measurement of the utilization of ingested calcium by three methods:

1. Determination of the amount of radiocalcium of the oral dose which passes unabsorbed as follows: oral dose — unabsorbed Ca^{45} = absorbed (utilized) Ca^{45}. This technique has been described in the previous communication (Blau et al., '54). For example, subject I.B. excreted 60% of the administered Ca^{45} in the stool in the first week after the ingestion of the isotope; of this 3% was endogenous and therefore 57% was unabsorbed and 43% was absorbed or utilized.

2. Measurement of endogenous fecal calcium as described in this communication, subtracting the endogenous fecal calcium from the stool calcium. The utilization is then calculated from the ratio of unabsorbed calcium to calcium intake.

3. The metabolic balance technique as used by Steggerda and Mitchell ('46), which is applicable only when balances at different intake levels are compared:

$$\% \text{ Utilization} = \frac{\text{Improvement of balance}}{\text{Change in intake}} \times 100.$$

Since three intake levels were studied in these two patients, the utilization was calculated in this manner.

TABLE 3

Endogenous fecal calcium, total digestive juice calcium, and utilization of dietary calcium

PERIODS [1]	CALCIUM INTAKE/DAY	S.A.[2] STOOL / S.A. URINE	ENDOGENOUS FECAL CALCIUM	TOTAL DIGESTIVE JUICE CALCIUM	UTILIZATION OF DIETARY CALCIUM IN PER CENT CALCULATED FROM		
					Ca^{45} balance	fecal calcium Endogenous	balance Metabolic
	mg		*mg/day*	*mg/day*			
			PATIENT I.B.				
1–4	107	0.60 [3]	91	160	43	44	..
5–8	529	0.21	91	140	..	40	40
9–12	1446	0.10	73	160	..	54	56
			PATIENT B.L.				
1–4	135	0.73 [3]	118	360	67	67	..
5–8	638	0.30	87	280	..	69	74
9–12	1740	0.13	93	240	..	61	59

[1] Each metabolic period consists of 6 days.

[2] S.A. = specific activity = % dose/100 mg Ca.

[3] Calculated from periods 2 to 4, after passage of unabsorbed dose of Ca^{45}.

The results obtained by these three methods are in remarkably good agreement for each intake level in both patients (table 3). I.B. absorbed and utilized less of the dietary calcium than B.L. The utilization of I.B. increased from 40 to over 54% when the calcium intake level was raised to the highest intake of 1446 mg of calcium per day. On the other hand, the utilization of dietary calcium in B.L. became less efficient on the highest intake of 1740 mg of calcium per day.

Minimum daily requirements. The urinary calcium and the endogenous fecal calcium are the two major avenues of body calcium loss, although some calcium loss also occurs through the sweat. Since the utilization was determined for both patients, it is possible to calculate the minimum daily requirements to cover such losses and to maintain calcium balance. For instance, the calcium losses of patient I.B. were 181 mg/day (urinary calcium 90 mg, endogenous fecal calcium 91 mg), the utilization was 42% (on low or intermediate calcium intake); therefore, a supply of 431 mg of calcium or 8.6 mg/kg body weight is required to cover these losses. Similar calculations for subject B.L. revealed a minimum calcium requirement of 240 mg/day or 3.5 mg/kg body weight. The minimum daily requirement can also be estimated from the data on metabolic balance by plotting the dietary intake against the balance and determining the intake level at which metabolic calcium equilibrium would result. Applying this method, a value of 450 mg was obtained for I.B., and 240 mg for B.L., which is in close agreement with the values calculated from the radiocalcium data. The advantages of the latter technique, however, are obvious, since the utilization can be calculated on one single intake level. The minimum calcium requirement for I.B. agrees with the figures cited in the literature (Sherman, '41; Cooper et al., '47) whereas it is lower in B.L. They do not suggest any increased calcium requirement in the aged.

SUMMARY

1. The endogenous fecal calcium, total digestive juice calcium and the utilization of ingested calcium have been measured in two adults at different levels of calcium intake.

2. Endogenous fecal calcium was found to be of the same order of magnitude as urinary calcium, 100 mg/day, and was independent of the level of calcium intake in both patients.

3. The maximum total digestive juice calcium was ~ 150 mg/day in one patient and ~ 360 mg/day in the other, values which are substantially lower than those estimated in the literature. Changes of the dietary calcium intake had little effect upon the total digestive juice calcium.

4. Approximately 45 and 65% of the dietary calcium was absorbed in the two patients respectively. Excellent agreement was found between the values obtained by three independent methods.

5. The minimum daily calcium requirements under the experimental conditions were calculated to be 8.6 mg/kg body weight for patient I.B. and 3.5 mg/kg body weight for patient B.L.

6. The values for the utilization of dietary calcium and the minimum dietary calcium requirements as calculated from the Ca^{45} data agree well with those obtained by the metabolic balance technique, confirming the validity of the radiocalcium calculations.

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LITERATURE CITED

BAUER, W., AND J. C. AUB 1927 Studies of inorganic salt metabolism. The ward routine and method. J. Am. Diet. Assn., *3:* 106.

BLAU, M., H. SPENCER, J. SWERNOV AND D. LASZLO 1954 Utilization and intestinal excretion of calcium in man. Science, *120:* 1029.

COOPER, L. E., E. M. BARBER AND H. S. MITCHELL 1947 Nutrition in Health and Disease, pp. 70. J. B. Lippincott Company, Philadelphia.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375.

HEVESY, S., L. HAHN AND O. REBBE 1939 Excretion of phosphorus. Kgl. Danske Videnskab. Selskab. Biol. Medd., *14:* 3.

KRAMER, B., AND F. F. TISDALL 1921 A simple technique for the determination of calcium and magnesium in small amounts of serum. J. Biol. Chem., *47:* 475.

LASZLO, D., C. A. SCHULMAN, J. BELLIN, E. D. GOTTESMAN AND A. SCHILLING 1952 Mineral and protein metabolism in osteolytic metastases. J. Am. Med. Assn., *148:* 1027.

NICOLAYSEN, R., N. EEG-LARSEN AND J. O. MALM 1953 Physiology of calcium metabolism. Physiol. Rev., *33:* 424.

SHERMAN, H. C. 1941 Chemistry of food and nutrition, pp. 254. The Macmillan Company, New York.

SHOHL, A. T., AND F. G. PEDLEY 1922 A rapid and accurate method for calcium in urine. J. Biol. Chem., *50:* 537.

STEGGERDA, F. R., AND H. H. MITCHELL 1946 Variability in the calcium metabolism and calcium requirements of adult human subjects. J. Nutrition, *31:* 407.

VISEK, W. J., R. A. MONROE, E. W. SWANSON AND C. L. COMAR 1953 Determination of endogenous fecal calcium in cattle by a simple isotope dilution method. Ibid., *50:* 23.

DIETARY FAT AND CHOLESTEROL METABOLISM

I. COMPARATIVE EFFECTS OF COCONUT AND COTTONSEED OILS AT THREE LEVELS OF INTAKE [1]

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Recent work has indicated that the kind and amount of fat eaten may influence absorption and storage of cholesterol and perhaps, also, its rate of synthesis (Hartroft, '56). The interpretation of data on fat intake is difficult, however, because it is often impossible to obtain accurate information concerning the composition of the dietary fat. The market for margarines and shortenings has become highly competitive. The manufacturer tends, therefore, to use the natural fat obtainable at the lowest cost, within the limitations of his label. Consequently, a shortening sold under a given brand name may have been made chiefly of soybean oil one week, and of coconut oil the next. If it is "blended" its chief constituent may be an inexpensive animal fat such as lard. Adjustments of melting point, texture and flavor are likely to be accomplished by such processes as partial hydrogenation, washing, chilling and pressing, and by the use of such additives as the law permits. Variants of potential importance in cholesterol metabolism are: kind and amount of sterol present; molecular weights and degree of unsaturation of the fatty acids; the

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formation of unnatural isomers during partial hydrogenation of the more unsaturated fatty acids; and the action of emulsants and other additives.

That some of the variations in composition of commercial shortenings are capable of producing differences in cholesterol storage in rats was indicated in our laboratory by the changed "base level" of liver cholesterol obtained in two replications of an experiment, carried out 6 months apart. The only known difference in diet was the use of two different batches of the same brand of a commercial vegetable shortening. A short study with lard, cottonseed oil, and a prepared shortening (Okey and Stone, '56) further indicated the need for a systematic investigation of the effect on cholesterol metabolism of the most frequently encountered changes in the composition of prepared food fats.

Studies with fats of known origin seemed the logical beginning. Levels of intake within "physiological" range for the experimental animal were obviously desirable for interpretation of results. Avoidance of the gross accumulation of liver glyceride which results from a very high fat intake was also important if cholesterol synthesis was to be measured.

Also, preliminary tests to find out whether the composition of the dietary fat was of any real importance in relation to cholesterol storage seemed to call for diets furnishing "low adequate," rather than highly lipotropic, intake levels of protein and choline. Data indicating that a diet furnishing 10% egg albumin and 5% casein (with 13.5% fat and 750 mg choline per kilogram diet) would support normal growth in young male rats were available from a previous study. This diet, which furnished 0.5 to 0.6% methionine, had not produced fatty livers when fed without cholesterol but, when fed with 1% cholesterol, it had not prevented accumulation of liver cholesterol ester. Protein-fat ratios in human dietaries have been found to be quite variable. Therefore it seemed likely that feeding 15% protein with several "moderate" levels of the first fats to be tested might yield pertinent information both as to the effect of the fats themselves and

as to the desirable ratios of either protein to fat or labile methyl to fat, or both for diets to be used for later dietary studies.

Two fats frequently used in margarines and shortenings were chosen: coconut oil, which is made up largely of glycerides of saturated fatty acids of comparatively low molecular weights, and cottonseed oil, which is rich in linoleic acid. The melting point of the latter was raised approximately to that of the coconut oil by the addition in the laboratory of about 10% of a completely hydrogenated cottonseed oil. This, presumably, meant the addition of the glycerides of fully saturated acids and eliminated the chance of formation of unnatural fatty acid isomers. The "plant sterol" content of each fat was low (under 0.3%). The fats were fed as 5, 10 and 15%, respectively, of the diets, and with and without the addition of 1% cholesterol. The 15% level of protein was used for all the diets. The consequent variation in proportion of dietary protein and fat calories and of choline was, however, recognized and evaluated in analysis of the lipid and growth data.

EXPERIMENTAL

General technique. Weanling rats of the Long-Evans strain were caged separately and given access to food and water at all times. Each group consisted of 10 males and 10 females. Weights and food intakes were recorded weekly. The composition of the diets is given in table 1; diets were kept in the freezer until fed. After 7 weeks on diet the rats were sacrificed by decapitation, and tissues and sera were prepared for analysis. Because fasting is known to retard cholesterol synthesis (Tomkins and Chaikoff, '52), and because some of the rats fed no added cholesterol were being used for measurements of cholesterol synthesis from acetate-C^{14}, the controls without cholesterol were allowed access to food until the time of autopsy. Food cups of the cholesterol-fed rats were, however, removed at 10 P.M. the evening preceding morning autopsy in order to avoid the variable increase in

serum cholesterol immediately following cholesterol ingestion noted by Ridout et al. ('54).

Liver samples to be used for lipid analysis were weighed and immediately homogenized in redistilled 95% alcohol. Serums were separated, frozen and stored until they could be analyzed. Fatty acids were determined by the method of Bloor ('28), and cholesterol by a modified Sperry and Webb ('50) procedure. Serum phospholipid was determined by the Sumner modification of the method of Fiske and SubbaRow (Sumner, '44).

TABLE 1

Composition of diets[1]

CONSTITUENT	AMOUNT		
	%	%	%
Casein	5	5	5
Egg albumin	10	10	10
Fat[2]	5	10	15
Sucrose	74	69	64
Salts (USP XIV)	4	4	4
"A" mix[3]	1	1	1
"B" mix[4]	1	1	1
Calories/100 gm	414	439	464
% cal. from fat[5]	13.0	22.6	31.0

[1] Diets for the groups of rats fed cholesterol were the same as for the corresponding control groups, except that 1% cholesterol was substituted for 1% sucrose.

[2] Iodine numbers of the fats were: coconut oil, 8; cottonseed oil (partially hardened, as fed), 97. The fats were prepared wthout additives through the courtesy of Mr. John E. Blum, Durkee and Company, Berkeley, California.

[3] The "A" mix furnished, per kilogram diet, 15,000 U.S.P. units "A" as distillate, 1000 U.S.P. units D, 5 mg menadione, 400 mg mixed tocopherol. It was made to volume with cottonseed oil, hence furnished about 5 gm linoleate per 1,000 gm diet.

[4] The "B" mix furnished in milligrams per kilogram diet: thiamine, 4; riboflavin, 4; pyridoxine, 2; calcium pantothenate, 10; folacin, 2; biotin, 1.5; *p*-amino-benzoic acid, 10; niacin, 10; ascorbic acid, 100; inositol, 500. Choline was incorporated into the diet separately at a level of 750 mg/kg.

[5] Including "carriers" for vitamins.

RESULTS AND DISCUSSION

Grams of food eaten, both by control and cholesterol-fed animals, tended to decrease slightly, and the caloric intake to increase slightly with the increase in percentage of fat in the diet (table 2). Mean variations in total intakes were less than 100 Cal. except for the control group of females fed 15% coconut oil, and the cholesterol-fed females on 10%

TABLE 2

Food intakes, weight gains, liver weights and liver lipids of rats fed coconut and cottonseed oils with and without cholesterol

DIET [1]		WEIGHT GAIN	FOOD INTAKE	LIVER WEIGHTS	LIVER LIPIDS
		gm	*gm*	*gm*	*% moist wt.*
Males					
CN	5%	205 ± 7.4 [2]	671 ± 14.7	10.1 ± 0.63	5.0 ± 0.20
	10%	221 ± 8.5	644 ± 18.3	10.6 ± 0.53	5.1 ± 0.26
	15%	227 ± 11.4	617 ± 21.7	13.1 ± 0.50	11.04 ± 2.58
CSO	5%	226 ± 11.8	672 ± 22.5	10.3 ± 0.51	3.98 ± 0.18
	10%	230 ± 15.3	665 ± 21.9	10.0 ± 0.56	4.90 ± 0.30
	15%	217 ± 11.6	641 ± 44.4	11.9 ± 0.77	9.9 ± 2.0
CN + C	5%	217 ± 10.3	667 ± 18.8	9.4 ± 0.47	6.5 ± 0.45
	10%	208 ± 7.1	651 ± 16.2	10.3 ± 0.63	11.1 ± 1.8
	15%	237 ± 11.1	634 ± 17.7	12.9 ± 0.92	22.6 ± 2.76
CSO + C	5%	227 ± 11.8	682 ± 27.4	11.5 ± 0.68	11.5 ± 0.87
	10%	217 ± 11.0	634 ± 27.5	14.3 ± 0.86	27.2 ± 1.50
	15%	192 ± 8.5	578 ± 21.4	16.3 ± 0.89	26.6 ± 1.60
Females					
CN	5%	147 ± 6.7	550 ± 14.2	6.9 ± 0.39	4.4 ± 0.14
	10%	141 ± 4.8	551 ± 11.0	6.8 ± 0.20	4.6 ± 0.18
	15%	170 ± 7.7	575 ± 19.6	8.2 ± 0.29	5.8 ± 0.45
CSO	5%	151 ± 5.7	554 ± 17.9	6.7 ± 0.41	4.45 ± 0.21
	10%	156 ± 4.8	554 ± 15.3	6.7 ± 0.36	4.2 ± 0.23
	15%	157 ± 4.5	556 ± 21.9	7.5 ± 0.23	4.8 ± 0.14
CN + C	5%	139 ± 5.5	550 ± 14.6	5.6 ± 0.17	5.9 ± 0.20
	10%	146 ± 6.9	543 ± 18.0	6.1 ± 0.19	6.1 ± 0.25
	15%	158 ± 6.4	513 ± 18.7	6.8 ± 0.33	11.0 ± 2.25
CSO + C	5%	146 ± 7.6	564 ± 15.1	6.2 ± 0.19	7.8 ± 0.49
	10%	137 ± 5.1	502 ± 11.4	6.3 ± 0.26	13.3 ± 1.70
	15%	169 ± 5.9	573 ± 17.7	10.4 ± 0.46	17.9 ± 1.90

[1] CN = coconut oil; CSO = cottonseed oil; CN + C = coconut oil + cholesterol.

[2] Mean ± standard error = $\sqrt{\frac{\Sigma d^2}{(n)\ (n-1)}}$.

cottonseed oil. With the exception of the males fed cottonseed oil, weight gains of all the 15% fat groups were somewhat greater than those for the rats with a lower percentage of fat in the diet. Differences were usually small and never of more than borderline significance because of variability within the groups. Also, while all the animals fed 5 and 10% fat were strictly comparable since they came from the same litters and were in the laboratory at the same time, limitations of space made it necessary to handle the rats fed 15% fat separately. Even small differences in laboratory temperature have been found to alter food consumption to the extent noted. On the whole, the ranges of the food intake and growth data could be taken to indicate that the animals of all the groups studied were comparable. Certainly none of them showed any signs of pathology other than accumulation of liver fat.

Liver weights in the control animals showed no significant increase with an increase of from 5 to 10% in dietary fat. There was always some increase in liver weight, however, and some gross appearance of lipid accumulation, when the fat intake reached 15%. This might be taken to indicate that the percentage of protein or choline or both in the 15% diets was borderline. With the addition of cholesterol to the diet, there was a stepwise rise in liver weight as fat consumption was increased. This was most marked in the males fed cottonseed oil, less so in those fed coconut oil. Females showed smaller differences than males.

Liver lipid percentages for the control rats fed both the 5% and the 10% levels of fat were well within normal ranges. With 15% of either fat, however, there was sufficient increase in liver lipid to suggest a marginal intake of lipotropic factors. When cholesterol was added to the diet differences in liver lipids between the rats fed coconut and cottonseed oil were evident at all levels of fat intake, and in both males and females. The fact that these differences were greater in growing males at the 10% level of fat intake than at 15% again suggested that the 15% diet might be marginal for

TABLE 3

DIET [1]		LIVER			SERUM	
		Total cholesterol		Free cholesterol [2]	Total cholesterol	Total phospholipid
		% moist wt.	*% fat free dry wt.*	*% moist wt.*	*mg %*	*mg %*
Males						
CN	5%	0.25 ± 0.006 [3]	0.88 ± 0.04	0.21	88.2 [4] ± 4.6	195 ± 9.9
	10%	0.27 ± 0.034	0.70 ± 0.11	0.22	72.4 [4] ± 5.0	185 ± 12.9
	15%	0.37 ± 0.072	1.45 ± 0.33	0.17	61.0 ± 4.8	167 ± 14.4
CSO	5%	0.226 ± 0.01	0.83 ± 0.06	0.20	76.0 [4] ± 3.5	178 ± 7.7
	10%	0.283 ± 0.03	1.04 ± 0.13	0.24	69.5 ± 3.6	152 ± 9.7
	15%	0.38 ± 0.04	1.38 ± 0.13	...	67.0 ± 3.2	
CN + C	5%	0.89 ± 0.08	3.42 ± 0.46	0.28	82.8 [4] ± 6.8	157 ± 10.9
	10%	1.45 ± 0.22	5.81 ± 0.99	0.38	96.2 ± 8.0	163 ± 11.6
	15%	2.27 ± 0.25	12.52 ± 1.49	0.34	77.9 ± 7.5	126 ± 15.3
CSO + C	5%	1.98 ± 0.26	7.9 ± 1.0	0.36	84.3 [4] ± 5.3	142 ± ...
	10%	3.33 ± 0.31	15.5 ± 2.0	0.55	54.7 ± 3.4	97 ± 6.6
	15%	3.73 ± 0.21	17.4 ± 2.1	...	82.0 ± 5.9	
Females						
CN	5%	0.23 ± 0.01	0.83 ± 0.004	0.21	83.0 ± 4.4	198 ± 8.7
	10%	0.25 ± 0.03	0.90 ± 0.10	0.21	85.8 ± 3.4	192 ± 12.3
	15%	0.23 ± 0.01	0.85 ± 0.04	0.20	71.9 ± 5.2	193 ± 5.9
CSO	5%	0.24 ± 0.01	0.89 ± 0.05	0.25	69.9 [4] ± 3.8	165 ± 10.3
	10%	0.27 ± 0.01	0.97 ± 0.11	0.22	69.7 ± 3.6	150 ± 7.6
	15%	0.26 ± 0.01	0.96 ± 0.04	...	78.0 ± 3.2	
CN + C	5%	0.63 ± 0.06	2.44 ± 0.24	0.36	101.0 ± 9.4	163 ± 7.3
	10%	0.89 ± 0.08	3.57 ± 0.36	0.44	128.0 ± 13.5	168 ± 7.1
	15%	1.72 ± 0.18	6.90 ± 0.82	0.35	129.0 ± 14.9	151 ± 7.9
CSO + C	5%	1.22 ± 0.13	5.30 ± 0.54	0.38	126.0 ± 21.4	133 ± 6.6
	10%	2.34 ± 0.36	10.0 ± 2.0	0.55	86.0 ± 12.2	130 ± 9.5
	15%	3.52 ± 0.28	16.0 ± 2.2	...	131.0 ± 11.4	

[1] CN = coconut oil; CSO = cottonseed oil; CN + C = coconut oil + cholesterol.

[2] Incomplete series.

[3] Mean ± standard error = $\sqrt{\frac{\Sigma d^2}{(n)\,(n-1)}}$.

[4] Excluding one atypical figure.

them. It is interesting that, even at a 5% intake level, cottonseed oil promoted liver storage of fat to a greater extent than coconut oil. The latter, as a fat rich in fatty acids of lower molecular weight, might be expected to be absorbed to the greater extent by way of the portal circulation.

Liver cholesterols (table 3) varied only slightly in control animals fed 5 and 10% of either fat. Control males fed 15% of either fat had rather high liver cholesterols, but values for females on these diets were not appreciably increased. When cholesterol was added to the diets, the liver cholesterols were much higher in the rats fed cottonseed oil than in those fed coconut oil at the corresponding levels. Contrasts were greatest for the groups fed 10% fat, but only slightly less so for those given only 5%. The smaller differences shown by the males fed the two fats at the 15% level again suggested complication due to a marginal proportion of labile methyl in the diet. The data as a whole indicate that the cottonseed oil may have been supplying the greater amount of some factor which promoted liver cholesterol storage.

Since a large proportion of the liver cholesterol stored by a rat on a cholesterol-rich diet is esterified, it seemed reasonable to assume that the active factors were specific fatty acids. Determination of free cholesterol in the livers of a considerable proportion of the control rats fed each fat at the 5% and the 10% levels revealed no very great differences between the groups fed coconut and those fed cottonseed oil. Nearly all of the cholesterol in the livers of the control rats was unesterified (means 0.17 to 0.25%), while the free cholesterol in the livers of those fed cholesterol ranged from 0.28 to 0.55% in test determinations, with values for the cottonseed oil rats tending to be slightly higher than for the coconut oil rats. The difference in the amount of liver cholesterol stored by the animals fed the two fats was, however, chiefly due to the larger amount of ester in the livers of those fed cottonseed oil. Alfin-Slater, Aftergood, Wells and Deuel ('54) have suggested that the esters of cholesterol with saturated (nonessential) fatty acids are less

readily available for normal metabolism than are those with essential fatty acids. Since the cottonseed oil, as fed, contained about 45% linoleic acid, while the coconut oil diets probably supplied a total of less than 1% (0.5% from the "A" mix), reconciliation of the present data with the preceding concept would require the assumption that accumulation of liver cholesterol ester is a normal step in cholesterol metabolism.

Mean total serum cholesterol values tended to be higher for rats fed 10% coconut oil than for those of the corresponding groups fed cottonseed oil. Differences were statistically significant for control males ($p < 0.01$), for cholesterol-fed males ($p < 0.01$), and probably for cholesterol females (p about 0.02). At the 5 and 15% levels of intake, differences were not significant but cholesterol-fed females with 5% fat showed the opposite trend.

Serum phospholipids showed some tendency to vary in the same direction as serum cholesterol. This might be taken to indicate a connection between the level of circulating cholesterol and that of phospholipid, and would be in line with the evidence that a large part of the circulating cholesterol is in combination in giant molecules also containing protein, phospholipid and fatty acid. Unfortunately, the amount of sample needed for the phospholipid determination did not leave sufficient for determination of free serum cholesterol.

The question of the ease with which different kinds of fatty acids esterify with cholesterol, and with which the esters are circulated and removed from the blood stream, obviously needs further investigation. If cholesterol's solubility in and esterification with absorbed fatty acid are factors in determining the amount entering the circulation, it might be expected that a diet rich in long-chain fatty acids (which, like cholesterol, enter the circulation largely by way of the lymph) would promote cholesterol absorption (Hernandez et al., '54). Short-chain fatty acids, which enter the portal circulation, might reasonably be expected to have the oppo-

site effect. The present data can be reconciled with this concept only if the extra linoleic acid supplied by the cottonseed oil also can be assumed to accelerate esterification and storage of cholesterol in the liver. The lower serum cholesterols observed in the rats fed 10% cottonseed oil, as compared with those fed 5%, are not irreconcilable with this premise.

The data indicate at least the possibility that, with a diet high in cholesterol, it may be of some importance whether the fat eaten is a margarine which consists chiefly of coconut oil or one made up of cottonseed oil. With an almost cholesterol-free diet, differences in composition of dietary fat had little effect on liver and serum lipids until the proportion of fat in the diet was increased to such a level that the adequacy of the lipotropic factors (protein, methionine and choline) was questionable.

In view of the high fat content of American diets, measurements with diets of higher fat content and increasing proportions of protein and choline are indicated, as are investigations with other food fats. Likewise, the possibility that qualitative as well as quantitative differences in the fat eaten may alter the supply of intermediates for cholesterol synthesis calls for study.

SUMMARY

Data are reported on lipid and cholesterol in liver and serum of rats fed, respectively, 5, 10, and 15% of coconut oil and of cottonseed oil slightly hardened by the addition of some of the completely hydrogenated fat, each with and without 1% cholesterol. When cholesterol was fed the linoleic acid-rich cottonseed oil led to more storage of liver cholesterol ester than did coconut oil. Ten per cent dietary coconut oil showed some tendency to produce higher serum cholesterol levels than did the same percentage of cottonseed oil. The data are discussed in relation to the specific effect of linoleic acid on mobility of cholesterol, and its preferential use in the formation and storage of cholesterol esters.

LITERATURE CITED

ALFIN-SLATER, R. B., L. AFTERGOOD, A. F. WELLS AND H. J. DEUEL, JR. 1954 The effect of essential fatty acid deficiency on the distribution of endogenous cholesterol in the plasma and liver of the rat. Arch. Biochem. Biophys., *52:* 180.

BLOOR, W. R. 1928 The determination of small amounts of lipid in blood plasma. J. Biol. Chem., *77:* 53.

HARTROFT, W. S. 1956 Arteriosclerosis and nutrition. Nutrition Rev., *14:* 193.

HERNANDEZ, H. D., I. L. CHAIKOFF, W. G. DAUBEN AND S. ABRAHAM 1954 The absorption of C^{14}-labeled epicholesterol in the rat. J. Biol. Chem., *206:* 757.

KIYASU, J. Y., B. BLOOM AND I. L. CHAIKOFF 1952 The portal transport of absorbed fatty acids. Ibid., *199:* 415.

OKEY, R., AND M. M. STONE 1956 Lard vs. vegetable fat: effects on serum and liver cholesterol. J. Am. Diet. Assoc., *32:* 807.

RIDOUT, J. H., J. M. PATTERSON, C. C. LUCAS AND C. H. BEST 1954 Effects of lipotropic substances on the cholesterol content of the serum of rats. Biochem. J., *56:* 306.

SPERRY, W. M., AND M. WEBB 1950 A revision of the Sperry-Schoenheimer method for cholesterol determination. J. Biol. Chem., *187:* 97.

SUMNER, J. B. 1944 A method for the colorimetric determination of phosphorus. Science, *100:* 413.

TOMKINS, G. M., AND I. L. CHAIKOFF 1952 Cholesterol synthesis by liver. I. Influence of fasting and of diet. J. Biol. Chem., *196:* 569.

ORGANIC FACTORS CONTROLLING THE EXCRETORY PATTERN OF POTASSIUM-42 AND CESIUM-134 IN RATS [1,2,3]

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Cesium, an alkali metal, has been reported by MacLeod and Snell ('50) to behave similarly to sodium, potassium and rubidium in the nutrition of lactic acid bacteria. Mraz et al. ('56) observed an increase in excretion of cesium-134 when the potassium content of the diet is increased. Hamilton ('45, '47a,b) reported that excretion of radio-cesium was relatively slow, requiring approximately 10 days to excrete 50%, with about equal removal in feces and urine. Hood and Comar ('53) reported a total cesium-137 excretion of 70% of the administered dose 7 days after administration of the nuclide, with 12 times more cesium-137 in the urine than in the feces.

In this laboratory differences in the excretory pattern of cesium-134 were first observed in studies dealing with the influence of potassium, sodium and hormones on cesium-134 metabolism. The rats fed a purified diet had a ratio of 25

[1] This manuscript is published with the permission of the director of the University of Tennessee, Agricultural Experiment Station, Knoxville, Tennessee.

[2] The radioactive materials used in this work were obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

[3] This work was completed under Contract no. AT-40-1-GEN-242 between the University of Tennessee, College of Agriculture, and the Atomic Energy Commission.

parts of urinary to one part of fecal cesium-134, while the rats fed diets containing natural foodstuffs excreted two to 6 parts of cesium-134 in the urine to one part in the feces. In the study to be presented the influence of natural foodstuffs on the excretory pattern of cesium-134 was tested first and those foodstuffs which produced excretory patterns considered of interest were later tried with potassium-42.

EXPERIMENTAL

The basal diet used during these studies had the following percentage composition: Casein 20, corn starch 63.2, cellulose 3, cottonseed oil [4] 4, vitamin concentrate 4, salt mixture 5 and potassium chloride 0.8. The vitamin concentrate supplied the following amounts of vitamins per kilogram of diet: vitamin A 25,000 I. U., vitamin D_3 7,500 I. C. U., thiamine 1.5 mg, riboflavin 23.5 mg, pyridoxin 1.5 mg, calcium pantothenate 56 mg, niacin 105 mg, *p*-aminobenzoic acid 30 mg, inositol 60 mg, folic acid 0.65 mg, and choline chloride 710 mg. The salt mixture was the potassium-deficient salt mixture used by Hove and Herndon ('55). The natural foodstuffs replaced part or all of the casein or starch in the basal diet.

Four weanling albino rats, uniform in size, were used per diet in each of the experiments to be reported on. Five microcuries of cesium-134 were administered subcutaneously to each rat in the cesium studies and a 72-hour balance study was made. The short half-life of potassium-42 (12.5 hours as compared to 2.3 years for cesium-134) necessitated the use of 200 microcuries of the nuclide and only a 48-hour balance study. Cesium-134 was used at tracer levels in the form of cesium chloride, while the potassium-42 dosage contained 26 mg of potassium in the form of potassium chloride All the rats were fed their respective diets for at least 7 days before administration of the nuclide. This period was considered of sufficient duration to permit the animal to adjust to the new diet. Longer feeding periods were used in some of

[4] Wesson oil.

the experiments in the interest of expediency. Excreta were digested with concentrated nitric acid, brought up to 25 ml with water and aliquots counted in a scintillation well-type counter.

In the interest of ease of recording and reading, fecal, urinary and total excretory data have been reported utilizing a maximum of two significant figures and are not indicative of the sensitivity of the experiments. The urinary to fecal ratios were obtained from data containing three significant figures and therefore differ slightly from the ratios which would be obtained from data reported here. The least significant differences reported for the ratios of urinary to fecal cesium-134 are crude measures of significance since variations within treatments having large ratios exert a disproportionately greater influence on the least significant differences than do variations within treatments having small ratios. The 5% level of confidence was the criterion for reporting significance.

The influence of the source of protein or carbohydrate upon the excretory pattern of cesium-134 was tested in experiment 1, table 1. In three groups, fish meal, soybean protein [5] and soybean oil meal replaced all of the casein in the basal diet and the starch content was manipulated so that the protein percentages derived from these materials were equal to that of the basal diet. In other groups, corn, oats and wheat replaced all of the starch in the basal diet while wheat flour comprised only 43% and wheat bran only 21% of the diet. The diets were fed for a period of 11 days before administration of the nuclide. In experiment 2, only the carbohydrate portion of the basal diet was varied. The foodstuffs and their percentages are shown in table 2. Seven per cent cellulose was included to determine if the fiber content of the oat hull diet was the sole factor in changing the ratio of urinary to fecal cesium-134. These diets were fed 7 days before administration of the nuclide. In experiment 3, those natural foodstuffs which had the greatest influence on the excretory

[5] Drackett Assay Protein. The Drackett Products Company, Cincinnati, Ohio.

TABLE 1

Influence of source of protein or carbohydrate upon the excretory pattern of cesium-134

TREATMENT	EXCRETION [1]			RATIO OF URINARY/FECAL Cs^{134}
	Urinary	Fecal	Total	
Supplement (% of diet)[2]				
Casein (20.0) — Starch (63.2)	10	0.3	10	38
Casein (20.0) — Corn (63.2)	13	0.5	13	28
Casein (20.0) — Oats (63.2)	15	2.6	18	6
Casein (20.0) — Wheat (63.2)	15	1.0	16	14
Casein (20.0) — Wheat flour (48.0)	11	0.3	11	32
Casein (20.0) — Wheat bran (16.0)	13	0.8	14	16
Fish meal (28.0) — Starch (55.2)	13	0.4	13	33
Soybean protein [3] (20.0) — Starch (63.2)	11	0.3	11	36
Soybean oil meal (40.0) — Starch (43.2)	24	2.2	26	11
L. S. D.[4]	3	0.4	3	21

[1] Expressed as percentage of subcutaneously administered dose per rat.

[2] Supplements replaced equivalent weights of cornstarch with the exception of fish meal, soybean protein and soybean oil meal which replaced all of the casein and 28, 0 and 20% cornstarch respectively.

[3] Drackett Assay Protein. The Drackett Products Company, Cincinnati, Ohio.

[4] Least significant difference between means at 5% level of confidence.

TABLE 2

Influence of source of carbohydrate upon the excretory pattern of cesium-134

TREATMENT	EXCRETION [1]			RATIO OF URINARY/FECAL Cs^{134}
	Urinary	Fecal	Total	
Supplement (% of diet)[2]				
Starch (63.2)	23	0.4	23	52
Rice (63.2)	18	0.4	18	43
Oats (63.2)	22	2.7	25	8
Rolled oats (43.0)	25	0.5	26	50
Oat hulls (21.0)	15	1.8	17	9
Alfalfa (21.0)	19	4.1	23	5
Cellulose (7.0)	20	0.9	21	23
L. S. D.[3]	6	0.9	6	24

[1] Expressed as percentage of subcutaneously administered dose per rat.

[2] Supplement replaced equivalent weight of cornstarch.

[3] Least significant difference between means at 5% level of confidence.

pattern of cesium-134 in the first and second experiments were tested using potassium-42. The diets shown in table 3 were fed for a period of 14 days before administration of potassium-42. The diets used in experiment 4, table 4, were fed for a period of 7 days before administration of cesium-134 to ascertain whether soybean oil meal, oat hulls and alfalfa were additive in their effect on the excretory pattern and whether the increased amount of dietary potassium introduced

TABLE 3

Influence of source of protein and carbohydrate upon the excretory pattern of potassium-42

TREATMENT	EXCRETION [1]			RATIO OF URINARY/FECAL K^{42}
	Urinary	Fecal	Total	
Supplement (% of diet)[2]				
Casein (20.0) — Starch (63.2)	11	0.3	11	34
Soybean oil meal (40.0) — Starch (43.2)	29	1.6	31	18
Casein (20.0) — Rolled oats (43.0)	19	0.4	19	43
Casein (20.0) — Oat hulls (21.0)	19	1.2	20	15
Casein (20.0) — Oats (63.2)	21	1.4	22	15
Casein (20.0 — Alfalfa (21.0)	24	1.5	25	16
L. S. D.[3]	10	0.7	10	21
L. S. D.[3] (Without rolled oats)				12

[1] Expressed as percentage of subcutaneously administered dose per rat.

[2] Supplements replaced equivalent weights of cornstarch with the exception of soybean oil meal which replaced all of the casein and 20% cornstarch.

[3] Least significant difference between means at 5% level of confidence.

by these foodstuffs influences the excretory pattern. Experiment 5 was initiated to ascertain if the factor in alfalfa which influenced the excretory pattern of cesium-134 was additive in its effect. The basal diet was slightly modified in both experiments 5 and 6 by increasing the cottonseed oil content to 8% at the expense of cornstarch in an attempt to make the high levels of alfalfa more palatable to the rats and to increase the energy content of the diets. Alfalfa replaced starch to the extent of 10, 20, 30, 40, 50 and 60% of the diet. These diets shown in table 5 were fed for a period of 7 days

before the nuclide was administered. The alfalfa diets in experiment 6 were designed to differ by increments of 20% to check the values obtained in experiment 5. Two additional diets were formulated to check the influence of the additional

TABLE 4

Influence of soybean oil meal, oat hulls, alfalfa and potassium on excretory pattern of cesium-134

TREATMENT	EXCRETION [1]			RATIO OF URINARY/FECAL Cs^{134}
	Urinary	Fecal	Total	
Supplement (% of diet) [2]				
Casein (20.0) — Starch (63.2) K (0.4)	19	1.1	20	17.5
Soybean oil meal (40.0) — Starch (43.2)	29	3.2	32	9.1
Casein (20.0) — Oat hulls (21.0)	23	2.7	26	8.6
Casein (20.0) — Alfalfa (21.0)	21	5.2	26	4.1
Casein (20.0) { Oat hulls (10.5) Alfalfa (10.5)	24	3.8	28	6.4
Casein (20.0) { Oat hulls (21.0) Alfalfa (21.0)	26	6.8	33	3.7
Soybean oil meal (40.0) { Oat hulls (10.5) Alfalfa (10.5)	32	7.9	40	4.0
Casein (20.0) — Starch (63.2), K (1.2)	26	1.1	27	24.6
L. S. D. [3]	4	0.9	4	2.0

[1] Expressed as percentage of subcutaneously administered dose per rat.

[2] Supplements replaced equivalent weights of cornstarch with the exception of soybean oil meal which replaced all the casein and 20% cornstarch.

[3] Least significant difference between means at 5% level of confidence.

protein supplied by the alfalfa added to the diets. Both these diets contained 15% of cottonseed oil to further increase the energy, one contained 40% alfalfa and 15% casein and the other contained 60% alfalfa and 10% casein. A 10% cellulose diet was also formulated to ascertain if the factor influencing the excretory pattern of cesium-134 was merely a function of

TABLE 5

Influence of dietary alfalfa levels on excretory pattern of cesium-134

TREATMENT	EXCRETION [1]			RATIO OF URINARY/FECAL Cs^{134}
% Alfalfa [2]	Urinary	Fecal	Total	
0	18	1	19	19.1
10	21	4	25	5.8
20	22	6	28	3.9
30	24	9	33	2.7
40	25	11	36	2.3
50	27	16	43	1.7
60	32	24	56	1.4
L. S. D.[3]	N. S.	4	11	3.5
L. S. D.[3] (For alfalfa groups)				1.7

[1] Expressed as percentage of subcutaneously administered dose per rat.

[2] Alfalfa replaced an equivalent weight of cornstarch.

[3] Least significant difference between means at the 5% level of confidence.

TABLE 6

Influence of dietary alfalfa levels, cellulose, oil and protein on the excretory pattern of cesium-134

TREATMENT	EXCRETION [1]			RATIO OF URINARY/FECAL Cs^{134}
	Urinary	Fecal	Total	
Supplements (% of diet)[2]				
Alfalfa (0)	17	1	18	12.3
Alfalfa (20)	22	4	26	5.4
Alfalfa (40)	31	10	41	3.2
Alfalfa (40)[3]	24	9	33	2.8
Alfalfa (60)	26	19	45	1.4
Alfalfa (60)[4]	27	17	44	1.6
Cellulose (10)	18	2	20	9.5
L. S. D.[5]	N. S.	6	7	4.5
L. S. D.[5] (For alfalfa groups)				1.2

[1] Expressed as percentage of subcutaneously administered dose per rat.

[2] Supplement replaced an equivalent weight of cornstarch.

[3] Oil content increased from a value of 8% to one of 15% and casein reduced from a value of 20% to one of 15%.

[4] Oil content increased to 15% and casein reduced to 10%.

[5] Least significant difference between means at the 5% level of confidence.

the fiber content of the diet. These diets shown in table 6 were fed for a period of 14 days before administration of the nuclide.

Studies of *in vitro* adsorption or formation of complexes were performed in 125 ml Erlenmeyer flasks employing 0.5 gm of air-dried alfalfa, wheat bran, soybean oil meal, oat hulls, cellulose, starch, casein or soybean protein and 25 ml of a cesium stock solution containing a 0.1 N solution of either potassium chloride, hydrochloric acid or sodium chloride. The flasks were shaken three times and allowed to stand 24 hours before aliquots of the supernatant solutions were removed.

RESULTS AND DISCUSSION

The influence of the source of carbohydrate and protein portions of the diet on the excretory pattern of cesium-134 may be observed in table 1. Of the carbohydrate sources fed, only the diets containing oats or wheat induced significantly higher fecal excretions of cesium-134 and lower ratios of urinary to fecal cesium-134 in rats than the basal diet containing cornstarch. The bran fraction of the wheat rather than the flour was responsible for this change in excretory pattern. Of the protein sources fed, only soybean oil meal exerted a significant influence on the excretory pattern of cesium-134. The significant differences observed in urinary excretion of cesium-134 might be explained by differences in the potassium contents of the natural foodstuffs fed.

In experiment 2, table 2, significant increases in fecal excretion of cesium-134 were observed in rats fed diets containing oats, oat hulls or alfalfa over those fed diets containing cornstarch, rice, rolled oats or cellulose. Lower urinary cesium-134 excretion was observed in rats fed the oat hull diet than in those receiving the diets containig cornstarch, oats or rolled oats. This decrease in urinary excretion of cesium-134 might be explained by the slightly lower growth rate experienced by those rats receiving the oat hull diet. The rats fed diets containing oats, oat hulls or alfalfa had significantly lower ratios of urinary to fecal cesium-134 than

did those fed diets containing cornstarch, rice or rolled oats. The rats fed the diet with added cellulose had significantly lower ratios than did those fed starch or rolled oats, but there were no significant differences among them in fecal excretion of cesium-134. Alfalfa and the hull fraction of oats did not appear to influence the excretory pattern of cesium-134 through their fiber content.

The knowledge obtained in experiments 1 and 2 with cesium-134 was applied in designing experiment 3, table 3, in which potassium-42 was administered. The significant differences in urinary excretion of potassium-42 might be explained by differences in potassium contents of the food-stuffs used in this experiment. Significantly more potassium-42 was found in the feces of rats fed diets containing soybean oil meal, oat hulls, oats or alfalfa than when the basal diet or the diet containing rolled oats was fed. The ratio of urinary to fecal potassium-42 was significantly higher in rats fed the diet containing rolled oats than in those fed diets containing soybean oil meal, oat hulls, oats or alfalfa. When the rats fed the rolled oats diet were eliminated from the statistical analysis, since very small differences in fecal potassium-42 exerted disproportionally greater differences in the ratio of urinary to fecal potassium-42, it was observed that rats fed the basal diet had a significantly greater ratio than did those fed diets containing soybean oil meal, oat hulls, oats or alfalfa. The natural foodstuffs used in the cesium-134 experiments seemed to exert a similar influence on the excretory pattern of potassium-42, but the differences between ratios of urinary to fecal potassium-42 were not as wide as occurred with cessium-134.

In experiment 4, table 4, significantly more cesium-134 was present in the feces of rats fed diets containing natural foodstuffs than in the feces of rats fed diets containing casein and starch as sole sources of protein and carbohydrate. The significant differences in urinary and total excretion of cesium-134 observed in rats fed different diets appeared to be a function of the potassium content of the diet whether

administered in the form of potassium chloride or in the natural foodstuff substituted for cornstarch. Total excretion was significantly lower in rats fed the basal diet than in those fed the other diets, while the ratio of urinary to fecal cesium-134 was greater in rats fed diets containing casein and starch than in those fed diets containing natural foodstuffs. Increasing the potassium content of the diet by inclusion of additional potassium chloride significantly increased the urinary and total excretion of cesium-134 and the ratio of urinary to fecal cesium but did not change the fecal excretion over that obtained on the basal diet. This indicated that the potassium contents of the natural foodstuffs were not responsible for decreases in the ratios of urinary to fecal cesium-134. The rats fed diets containing alfalfa had significantly lower ratios than did those fed the other diets. Oat hulls, soybean oil meal and alfalfa appeared to be additive in their influence on the excretory pattern of cesium-134.

The level of alfalfa in experiment 5, table 5, did not significantly influence urinary excretion of cesium-134, but significant differences were observed in fecal and total excretion of cesium-134. The trend toward greater total excretion of cesium-134 as the alfalfa level increased was probably a function of the potassium content of the alfalfa. Total excretion of cesium-134 was observed to increase threefold over the range of from zero to 60% alfalfa, while fecal excretion increased 26-fold over the same range. Rats fed diets containing alfalfa had significantly lower ratios than did those fed the basal diet. When only the alfalfa-containing diets were compared, the rats fed the 10% alfalfa diet had a significantly greater ratio than did those fed the other alfalfa-containing diets. The rats fed diets containing 50 and 60% alfalfa had significantly lower ratios than did those fed diets containing 20% or less alfalfa. The ratios varied inversely with the alfalfa contents of the diets.

The 10% cellulose diet fed in experiment 6, shown in table 6, did not significantly increase fecal or total excretion of cesium-134 or decrease the ratio of urinary to fecal cesium

over that obtained on the basal diet, while diets containing alfalfa did. When only the rats on the alfalfa-containing diets were compared, a significant difference in the ratios of urinary to fecal cesium-134 was observed between each level of alfalfa fed. Raising the oil content of the 40 and 60% alfalfa diets from a level of 8% to one of 15% and adjusting the casein to allow for the protein introduced by the alfalfa did not significantly change fecal excretion of cesium-134 or the ratio of urinary to fecal cesium. The 5th and 6th experiments indicate that the factor in alfalfa which influences the excretory pattern is additive and is not purely a function of the potassium, cellulose or protein level of the alfalfa added to the diet.

It was found in the *in vitro* studies that cellulose, cornstarch, casein and soybean protein did not influence the cesium-134 in solution while alfalfa removed 6, 8 and 15% of the cesium-134 out of the 0.1 N solutions of potassium chloride, hydrochloric acid and sodium chloride, respectively. Wheat bran, soybean oil meal and oat hulls were observed to remove about 2 to 3% of the cesium-134 out of these solutions. When these three foodstuffs were tested *in vivo* there were no significant differences between the ratios of urinary to fecal cesium-134 while alfalfa showed a tendency to reduce this ratio by a factor of 2 or 3 below that exhibited by these three foodstuffs. The *in vitro* data appear to indicate that alfalfa, oat hulls, soybean oil meal and wheat bran influence the excretory pattern of cesium-134 through adsorption of the nuclide in the gut as it is recirculated through the body.

SUMMARY

Experiments were conducted to test the influence of natural foodstuffs on the excretory pattern of cesium-134 and potassium-42. The experiments demonstrated that oat hulls, wheat bran, alfalfa meal and crude soybean oil meal influence the excretory pattern of cesium-134 and potassium-42 in rats by increasing the excretion of these nuclides in the feces as

compared to their pattern of excretion when corn, cornstarch, rolled oats, wheat flour, casein, fish-meal or a purified soybean protein, are fed. This effect is not explained by the potassium, cellulose nor protein level in the natural foodstuffs fed, but appears to be an adsorption phenomenon.

LITERATURE CITED

HAMILTON, J. G. 1945 Tracer studies of fission product metabolism. MDDC, 1062. Atomic Energy Commission. February 2.

———— 1947a The metabolism of fission products and the heaviest elements in rats and plants. MDDC, 1160. Atomic Energy Commission, June 11.

———— 1947b The metabolism of the fission products and the heaviest elements. Radiology, *49:* 325.

HOOD, S. L., AND C. L. COMAR 1953 Metabolism of cesium-137 in rats and farm animals. Arch. Biochem. Biophys., *45:* 423.

HOVE, E. L., AND J. F. HERNDON 1955 Potassium deficiency in the rabbit as a cause of muscular dystrophy. J. Nutrition, *55:* 363.

MACLEOD, R. A., AND E. E. SNELL 1950 The relation of ion antagonism to the inorganic nutrition of lactic acid bacteria. J. Bacteriol., *59:* 783.

MRAZ, F. R., M. LENOIR, J. PINAJIAN AND H. PATRICK 1956 Influence of potassium and sodium on uptake and retention of cesium-134 in rats. Abstract, Conference of Radioactive Isotopes in Agriculture, East Lansing, Michigan, January 12–14, p. 3.

THE ADDITION OF SMALL AMOUNTS OF DEFATTED FISH FLOUR TO MILLED WHEAT FLOUR, CORN MEAL AND RICE

INFLUENCE ON GROWTH AND PROTEIN EFFICIENCY [1]

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Inasmuch as the sea offers an abundance of good quality proteins (Lanham and Lemon, '38; Nilson, Martinek and Jacobs, '47; Finn, '54; Walford and Wilber, '55), it was thought of interest to investigate the influence on growth and protein efficiency of additions of small amounts of fish flour to the proteins in enriched milled wheat flour, white corn meal, and polished rice. This was of particular interest since the Food and Agriculture Organization of The United Nations and the United Nations International Children's Emergency Fund are now introducing fish flour into human dietaries in the battle against kwashiorkor. For a preliminary study UNICEF furnished this laboratory with a large supply of dehydrated but non-defatted fish flour from Valparaiso, Chile. Such a product with a fat content of 9.9%, has poor keeping qualities and becomes rancid in warm weather, but the encouraging results obtained with it warranted a continuation of the study with defatted fish flour, containing less than one per cent fat.[2] The fish used for dehydrating

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[2] Supplied by the Viobin Corporation, Monticello, Illinois.

and defatting was a mixture of carp, smelts and whitings. The fish product as furnished was extracted with ethylene dichloride and dehydrated at a temperature of 71°C. at atmospheric pressure by the process of Levin ('52); Levin and Finn ('55). The fish flour thus produced contained 78% protein; 0.4% fat; 4.6% water; and 16.9% minerals.

EXPERIMENTAL

This study was carried out on the Wistar strain albino rats. There were 12 animals in each group, the sexes being equally represented. The animals were about 28 days old when started on experiments and weighed 50 to 53 gm each. The experimental period was 10 weeks on the wheat and rice rations and 6 weeks on the corn rations. The animals were weighed once weekly and accurate records kept of food consumption. The protein content of the milled wheat flour was 9.9%; of the corn meal, 7.8% and of the polished rice, 6.1%. It was possible to incorporate in the basal rations enough of the milled grains to provide 8% of protein from milled wheat flour, 6% from the cornmeal and 5% from polished rice. Fish flour was supplied at three levels, 1, 3 and 5%, at the expense of the milled grain. The balance of the rations consisted of celluflour 2%, Sure's salts no. 1 ('41) 4%, vegetable shortening 7%, cod liver oil 2%, wheat germ oil 1% and glucose to make 100%. All rations contained an abundance of the B vitamins (Sure, '53, '54). The fat-soluble vitamins A, D and E were furnished by the cod liver oil and wheat germ oil in the rations. The results of this investigation are summarized in tables 1, 2 and 3 and are expressed as gains in body weight per gram of protein intake, which indicates the protein efficiency ratio (PER).

The results given in the tables are self-explanatory. On the enriched milled wheat flour rations, the addition of 3% of fish flour resulted in optimum protein efficiency, although maximum growth, namely, a 7-fold increase in body weight,

[3] Cerelose.

TABLE 1

The addition of small amounts of defatted fish flour to enriched milled wheat flour. Influence on growth and protein efficiency

(Experimental period was 10 weeks, with 12 rats in each group. Results are means)

TYPE OF RATION	AMOUNT IN RATION	PROTEIN	GAINS IN BODY WEIGHT		TOTAL FOOD INTAKE	PROTEIN INTAKE	PROTEIN EFFICIENCY RATIO [1]	
				Increase				Increase
	%	%	gm	%	gm	gm		%
Milled wheat flour	80.7	8.0	23.4 ± 4.9 [2]	....	413.7	33.1	0.71 ± 0.04 [3]	....
Milled wheat flour Defatted fish flour	79.7 1.0	8.69	53.9 ± 5.6	130.3	554.9	48.1	1.12 ± 0.06	57.7
Milled wheat flour Defatted fish flour	77.7 3.0	10.05	124.0 ± 8.3	439.1	755.2	75.4	1.65 ± 0.05	132.4
Milled wheat flour Defatted fish flour	75.7 5.0	11.41	159.2 ± 9.5	580.3	831.0	98.9	1.61 ± 0.07	126.7

[1] Expressed as gains in body weight per gram of protein intake.
[2] Standard deviation.
[3] Standard deviation of the means.

TABLE 2

The addition of small amounts of defatted fish flour to milled white corn meal. Influence on growth and protein efficiency

(Experimental period was 6 weeks, with 12 rats in each group. Results are means)

TYPE OF RATION	AMOUNT IN RATION	PROTEIN	GAINS IN BODY WEIGHT		TOTAL FOOD INTAKE	PROTEIN INTAKE	PROTEIN EFFICIENCY RATIO [1]	
				Increase				Increase
	%	%	gm	%	gm	gm		%
Milled white corn meal	76.0	6.0	4.6 ± 2.3 [2]	....	239.2	14.4	0.32 ± 0.03 [3]	....
Milled white corn meal Defatted fish flour	75.0 1.0	6.70	25.2 ± 4.6	447.8	288.7	19.3	1.31 ± 0.04	309.4
Milled white corn meal Defatted fish flour	73.0 3.0	7.82	38.9 ± 5.3	745.6	318.7	25.6	1.52 ± 0.06	375.0
Milled white corn meal Defatted fish flour	71.0 5.0	9.50	69.5 ± 6.9	1,410.9	394.7	37.1	1.87 ± 0.08	484.4

[1] Expressed as gains in body weight per gram of protein intake.
[2] Standard deviation.
[3] Standard deviation of the means.

was secured with the addition of 5% of fish flour (table 1). The corn meal used was apparently an overmilled product, as indicated by the small gains in body weight secured on the basal ration. There were enormous increases in body weight and protein efficiency with the increase in fish flour intake. On the addition of 5% of fish flour there was a 15-fold increase in growth (table 2). Corn is a significant cereal grain for human consumption in the southern part of the United States, in Guatemala, where it supplies about 70% of the total protein intake (Scrimshaw, '56), and in South Africa, Yugoslavia, and Mexico (U.S.D.A. Year Book, '51) but its proteins have a very low biological value, being deficient in the essential amino acids lysine, tryptophan, methionine, threonine (Sure, '53) and isoleucine (Sauberlich, Chiang and Salmon, '53). It would seem that small additions of defatted fish flour to the high corn-containing diets in these countries should prove of material benefit in reducing the prevalence of kwashiorkor. This possibility should, of course, be explored by suitable clinical trials.

That fish flour is acceptable in the human diet has been recently demonstrated in Chile (Sure, '56). A bread containing 10% of fish flour had excellent acceptance among 140 school children. Compared with normal bread, the only difference was a slightly darker color, the smell, taste, form and consistency of the crust being normal. There was not a single rejection or complaint, and no digestive trouble traceable to the bread occurred. The same type of bread fortified with fish flour was given to young men in a military camp. Here also this type of bread was very well accepted.

With the polished rice rations, the addition of 1% of fish flour produced optimum protein efficiency, although the further increase to 3 and 5% of fish flour resulted in increased growth (table 3). It was previously demonstrated in this laboratory that, while rice is low in nitrogen, the quality of the proteins is nevertheless the best of the cereal grains (Sure, '48a); this is confirmed by the fact that the animals

TABLE 3

The addition of small amounts of defatted fish flour to polished (milled) rice. Influence on growth and protein efficiency

(Experimental period was 10 weeks, with 12 rats in each group. Results are means)

TYPE OF RATION	AMOUNT IN RATION	PROTEIN	GAINS IN BODY WEIGHT		TOTAL FOOD INTAKE	PROTEIN INTAKE	PROTEIN EFFICIENCY RATIO [1]	
				Increase				Increase
	%	%	gm	%	gm	gm		%
Polished (milled) rice	84.0	5.0	41.2 ± 5.8 [2]	....	535.8	26.8	1.54 ± 0.05 [3]	...
Polished (milled) rice	83.0	5.86	90.6 ± 7.3	119.9	636.3	36.2	2.50 ± 0.09	62.3
Defatted fish flour	1.0							
Polished (milled) rice	81.0	7.30	132.6 ± 8.1	221.8	744.1	52.8	2.51 ± 0.08	64.3
Defatted fish flour	3.0							
Polished (milled) rice	79.0	8.73	151.0 ± 8.9	281.1	776.2	65.9	2.39 ± 0.09	55.2
Defatted fish flour	5.0							

[1] Expressed as gains in body weight per gram of protein intake.

[2] Standard deviation.

[3] Standard deviation of the means.

on the basal polished rice rations made the best growth. On the 5% level of fish flour the body weight gains were a little better than 3½ times those obtained with the basal ration. These results may have wide applications in large sections of the world, particularly in overpopulated Asia where rice provides, in certain regions, 80 to 85% of the caloric intake as well as the greater proportion of the protein of the diet.

The defatted fish flour added as 1, 3, and 5% in the rations proved a much more efficient protein supplement to the proteins in milled cereal grains than dried non-fat milk solids, dried buttermilk, dried brewers' yeast and cultured food yeasts, defatted soybean flour or peanut meal (Sure, '46, '47, '48a). As an illustration, the addition of 1, 3 and 5% of cultured food yeast, as supplements to the proteins in polished rice, produced increases in body weight of 41.3, 59.1, and 69.0%, respectively, while the same amounts of defatted fish flour produced increases of 119.9, 221.8, and 281.1%; also, 1% of food yeast as a supplement to the proteins in polished rice resulted in an increase of PER of 15.7 (Sure, '46) while with 1% defatted fish flour there was an increase of 62.3 in PER. In comparing the relative values of small amounts of defatted fish flour and other high-protein-containing foods (Sure, '48b) as supplements to the proteins in cereal grains it should be taken into consideration that fish flour has a very high protein content and a high biological value, while the proteins in soybean and peanut meals, at low levels of protein intake have low biological values (Sure, '55). The beneficial effects of the addition of small amounts of fish flour to the proteins in milled cereal grains may be due also, in part, to their contribution of vitamin B_{12} (Peeler et al., '51).

SUMMARY

A study was made of the influence of additions of small amounts of defatted fish flour to the proteins in milled wheat flour, white corn meal, and polished rice. The enormous gains in body weight and protein efficiency obtained were

far superior to those secured in the past with dried non-fat milk solids, dried butter-milk, defatted soybean flour, brewers' yeast, cultured food yeasts, and peanut meal. Such results may prove of material assistance in combating protein deficiency diseases such as kwashiorkor, which is prevalent among infants and young children in Asia, Africa, and Latin American countries. Such applications of these findings with laboratory animals should be explored by suitable clinical trials.

LITERATURE CITED

FINN, D. B. 1954 The sea and world food supplies. Nutrition Abstr. and Rev., *24:* 487.

LANHAM, W. B., AND J. M. LEMON 1938 Nutritive value for growth of some proteins of fisherly products. Food Research, *3:* 549.

LEVIN, E. 1952 Drying and Defatting Tissue. U. S. Patent No. 2,619,425, Nov. 25, 1952.

LEVIN, E., AND R. K. FINN 1955 A process for dehydrating and defatting tissues at low temperature. Chem. Eng. Progress, *51:* 223.

NILSON, H. W., W. A. MARINEK AND B. JACOBS 1947 Nutritive value for growth of some fish proteins. U. S. Dept. of Interior Fish and Wildlife Service, Sep. 178, *9:* No. 7, 1.

PEELER, H. T. H., H. YACOWITZ, C. W. CARLSON, R. F. MILLER, L. C. NORRIS AND G. F. HEUSER 1951 Studies on the vitamin B_{12} content of feedstuffs and other materials. J. Nutrition, *43:* 49.

SAUBERLICH, H. W., W. CHIANG AND W. D. SALMON 1953 The comparative nutritive value of corn of high and low protein content for growth in the rat and chick. Ibid., *51:* 623.

SCRIMSHAW, N. S. Personal communication.

SURE, B. 1941 The existence of a new dietary factor essential for lactation. J. Nutrition, *22:* 499.

———— 1946 Nutritional improvement of cereal flours and cereal grains. J. Am. Dietet. Assoc., *22:* 494.

———— 1947 Further studies on nutritional improvement of cereal flours and cereal grains with yeast. Ibid., *23:* 113.

SURE, B., AND F. HOUSE 1948a The relative biological value of the proteins in cereal grains. J. Nutrition, *36:* 595.

SURE, B. 1948b Relative supplementary values of dried food yeasts, soybean flour, peanut meal, dried non-fat milk solids, and dried buttermilk to the proteins in milled white corn meal and milled enriched wheat flour. Ibid., *36:* 75.

———— 1953 Improvement in whole yellow corn with lysine, tryptophan, and threonine. J. Agr. and Food Chem., *1:* 626.

SURE, B. 1954 Relative nutritive values of proteins in whole wheat and whole rye and effect of amino acid supplements. Ibid., *2:* 1108.

———— 1955 Relative nutritive values of proteins in foods and supplementary value of amino acids in pearled barley and peanut flour. Ibid., *3:* 789.

———— 1956 The critical world food situation. Am. J. Clin. Nutrition, *4:* 220.

U. S. DEPT. AGR. YEARBOOK OF FOOD AND AGRICULTURAL STATISTIC PRODUCTION 1951 Table II, p. 23.

WALFORD, L. A., AND G. WILBER 1955 The sea as a potential source of protein food. Advances in Protein Chemistry, *10:* 289.

COMPARISON OF AMINO ACID MIXTURES AND EGG PROTEIN AS SOURCES OF DIETARY NITROGEN [1]

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In a series of papers from this laboratory (Anderson and Nasset, '48, '50; Nasset and Anderson, '50, '51; Nasset, Anderson and Siliciano, '51; Nasset and Siliciano, '52; Nasset and Ely, '52, '53; Nasset and Gatewood, '54) nitrogen balance data were presented which were obtained from adult male rats fed amino acid diets. From these data the mean minimum requirement of each essential amino acid for the maintenance of nitrogen equilibrium was computed. These requirements were determined singly for each of 9 essential amino acids (arginine is not essential for nitrogen balance in the adult rat) in experiments in which the concentration of the amino acid in question was the only experimentally imposed variable. The limiting concentration of each essential amino acid, therefore, was determined in the presence of a relative excess of the others. A most interesting point is whether the mean minimum requirements determined individually in this manner can serve as a model for a new, and presumably highly efficient, mixture of amino acids as a source of dietary nitrogen.

Apparently the results of feeding such a mixture have not previously been published. The nearest approach to this

[1] This investigation was financed in part by grants from the Office of Naval Research and the Fluid Research Fund of the School of Medicine and Dentistry, University of Rochester.

type of experiment was reported by Rose and Wixom ('55). They fed two adult human males a mixture which provided "safe" quantities of 8 essential amino acids with various amounts of glycine and urea as sources of nitrogen for the synthesis of non-essential amino acids. The "safe" quantity of any essential amino acid was taken arbitrarily by these investigators to be twice the amount needed by the subject who, in previous experiments, required the most for the maintenance of "distinctly positive" nitrogen balance. This state of nitrogen balance was not defined but for the two subjects studied it was presumably + 0.15 gm N/day. Leverton et al ('56a, b, c, d, e) fed a mixture which supplied the minimum requirements of threonine, valine, tryptophan, phenylalanine and leucine to adult human females. They related their results to a "nitrogen equilibrium zone" in which nitrogen excretion, except for two subjects, remained within the limits of 95 to 105% of nitrogen ingestion.

The work described in the present paper was done in order to test the relative adequacy of isonitrogenous quantities of whole egg and two mixtures of amino acids in maintaining nitrogen equilibrium in the rat.

METHODS

A single group of adult male albino rats (Wistar strain) served for all of these experiments, which extended over a period of 25 weeks. After an initial 48-hr. fast the body weights ranged from 216 to 288 gm (mean 257 gm). The rats were housed individually in metabolism cages kept in a windowless room in which the temperature was maintained between 26 and 28°C. Alternating 12-hr. periods of darkness and light were provided by means of a switch operated by a clock.

The composition of 5 diets is shown in table 1, the last three of which are essentially isonitrogenous and isocaloric. The composition of the two amino acid mixtures is given in table 2. The crystalline amino acids were obtained com-

cercially [2] and the purity of each lot was confirmed by nitrogen analyses and in some instances also by measurement of specific rotation. The "complete" mixture simulates whole egg to the extent that it contains approximately the same quantity as whole egg of each L-isomer of the essential amino acids per gram of total nitrogen. The non-essential amino acids are replaced in this mixture by the D-isomers of 6 essential amino acids plus L-glutamic acid. The "new" mixture

TABLE 1

Composition of 100 gm of diet [1]

DIETS	MAINTENANCE	N-FREE	"COMPLETE" AA [2]	"NEW" AA [3]	WHOLE EGG
	%	%	%	%	%
Dried whole egg [4]	20.25	0	0	0	6.67
Amino acid mixture	0	0	4.17	5.51	0
Sucrose	59.96	70.49	66.22	66.15	66.57
Cottonseed oil	8.45	17.40	17.46	17.46	14.59
Si O_2 or Cr_2O_3 marker	1.94	2.31	2.32	2.32	2.32
Salt mixture (Wesson)	4.51	4.50	4.51	4.51	4.51
Cellulose flour	4.89	5.30	5.32	4.05	5.34
Total nitrogen	1.54	0	0.52	0.53	0.51
Physiological heat value	431 Cal.	437 Cal.	437 Cal.	442 Cal.	435 Cal.

[1] Each 100 gm of diet also contained the following: 0.92 mg thiamine chloride, 0.92 mg pyridoxine hydrochloride, 1.84 mg riboflavin, 4.6 mg niacin amide, 5.1 mg calcium pantothenate, 0.06 mg folic acid, 0.06 mg biotin, 115 mg choline chloride, 0.001 mg vitamin B_{12}, 1.15 mg methyl naphthoquinone, 4.6 mg α-tocopherol, 7280 I.U. vitamin A, 1456 I.U. vitamin D.

[2,3] Composition of amino acid mixtures given in table 2.

[4] Total N, 7.61%; total lipid, 42.5%.

is designed to supply each essential amino acid in the relative proportions as well in the absolute amounts suggested by the mean minimum requirements of the essential amino acids as previously determined individually in this laboratory from 1948 to 1954. Neither of these mixtures contained cystine, cysteine or tyrosine.

Each experiment included the following 5-week cycle of feeding: maintenance diet (9.6% whole egg protein), first

[2] Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE 2

Amino acids per 100 gm of diet

"COMPLETE" AMINO ACID MIXTURE						"NEW" AMINO ACID MIXTURE			
	Grams	Millimoles		Mole ratios [1]			Grams	Milli-moles	Mole ratios
		Actual [2]	Available [3]	Actual	Available				
L-Arginine·HCl	0.377	1.79	1.79	0.67	1.33	L-Arginine·HCl	0	0	0
L-Histidine·HCl·H_2O	0.128	0.62	0.62	0.23	0.46	L-Histidine·HCl·H_2O	0.025	0.12	0.20
DL-Isoleucine	0.445	3.39	1.70	1.26	1.26	L-Isoleucine	0.108	0.82	1.37
L-Leucine	0.310	2.36	2.36	0.88	1.75	L-Leucine	0.058	0.44	0.73
L-Lysine·HCl	0.257	1.41	1.41	0.52	1.04	L-Lysine·HCl	0.016	0.09	0.15
DL-Methionine	0.248	1.66	1.66	0.62	1.23	L-Methionine	0.144	0.96	1.60
DL-Phenylalanine	0.394	2.38	2.38	0.88	1.76	L-Phenylalanine	0.065	0.39	0.65
DL-Threonine	0.320	2.69	1.35	1.00	1.00	L-Threonine	0.072	0.60	1.00
DL-Tryptophan	0.108	0.53	0.35	0.20	0.26	L-Tryptophan	0.022	0.11	0.18
DL-Valine	0.467	4.00	2.00	1.49	1.48	L-Valine	0.065	0.56	0.93
L-Glutamic acid	1.112	7.57	7.57	2.81	5.60	L-Glutamic acid	4.930	33.5	55.8
Total weight of AA	4.166					Total weight of AA	5.505		

[1] On the basis of threonine taken as unity.

[2] Actual amounts present regardless of optical isomers.

[3] Based on availability of D-isomers as shown in previous work from this laboratory. No experiments were done with D-isoleucine but judging from the work of others it is assumed to be unavailable. Under conditions of these experiments, racemic methionine and phenylalanine are equivalent to the natural form, the D-isomers of threonine and valine are unavailable, and 1.0 gm of L-tryptophan is equivalent to 1.5 gm of DL-tryptophan.

and second weeks; nitrogen-free (N-free) diet, third week; amino acid or protein-containing diet which supplied approximately half of the nitrogen required for nitrogen equilibrium (half-N), 4th week; amino acid or protein-containing diet which contained twice the amount of nitrogen in the half-N diet (full-N), 5th week. It was assumed that two weeks on maintenance diet (low protein content) followed by one week on N-free diet would serve to induce a uniform state of protein depletion before the feeding of test diets was begun. No quantitative measure is available, however, to justify this assumption.

In order to assure complete consumption of diets they were fed by stomach tube. The maintenance diet, however, was weighed out daily in the first two weeks of each feeding cycle and consumed voluntarily. In this way it was possible to maintain the usual energy intake of 121 Cal./day/kg[3] and the usual total nitrogen intake of 140 to 150 mg/day/kg[3]. To prevent clogging of the stomach tube the dry components, except sucrose, were first finely ground (Wiley mill, 60 mesh screen) and then the whole diet was mixed with water to give a final water content of 30% before being homogenized. Feeding was done twice each day and samples were delivered from the stomach tube into weighing bottles at the beginning and end of each feeding. Thus 4 samples were collected each day and analyzed for total nitrogen. In addition the individual dry components as well as the dry diet mixture were analyzed for nitrogen.

Excreta were collected for analysis in the last two weeks of each feeding cycle. Feces were collected daily and stored in sulfuric acid. They were marked by adding powdered Cr_2O_3 to the first feeding of each 7-day period. In order to maintain the same quantity of inert material in the diet, SiO_2 was substituted for Cr_2O_3 after the first feeding. The change in color was very sharp and both of these compounds seemed to be quite inert. Urine was collected for analysis during the last 4 days of each period. On the first and third of these days the stainless steel urine funnel was washed

with hot water and finally sprayed with 10 ml of 50% ethanol, containing 30 gm of benzoic acid and 0.5 gm/l of phenyl mercuric nitrate. The excess drained into the flask below and served as an excellent preservative.

RESULTS

Average results of all experiments are contained in table 3. The body weights given are averages of the individual body

TABLE 3

Average data for rats receiving diets by stomach tube

(Series 440 rats)

EXPERIMENT NUMBER	I	II	III	IV	V
DIET	"Complete" AA mix	"New" AA mix	Whole egg	"New" AA mix	Whole egg
Number of rats	13	14	12	13	16
Body weight (kg)	0.248	0.245	0.244	0.260	0.259
Metabolic body size ($kg^{3/4}$)	0.351	0.349	0.347	0.363	0.362
N balance data (mg N/day/$kg^{3/4}$)					
Half-N periods:					
Food nitrogen	74	81	85	66	73
Fecal nitrogen	43	43	42	34	38
Urinary nitrogen	145	149	109	115	109
Full-N periods:					
Food nitrogen	146	148	148	136	141
Fecal nitrogen	43	41	50	34	41
Urinary nitrogen	125	179	97	141	91
Nitrogen balance	−23	−72	+1	−39	+10
NI_e[1]	166 ± 4	288 ± 16	148 ± 3	203 ± 8	134 ± 2

[1] Computed nitrogen intake required for nitrogen equilibrium.

weights at the end of the N-free, half-N, and full-N periods of each 5-week feeding cycle. An inexplicable gain of approximately 6% occurred in experiment IV which was maintained in experiment V (16th to 25th week), but otherwise average body weights remained constant. Fecal nitrogen within each experiment was reasonably constant. Urinary nitrogen excretion conformed to the usual pattern observed repeatedly in earlier experiments of this series. In passing

from the half-N period to the full-N period the nitrogen intake was doubled. When the animals received egg protein or the "complete" amino acid mixture the urinary nitrogen was always slightly diminished on doubling the nitrogen intake. When the animals received double the amount of the "new" amino acid mixture (exps. II and IV) the excretion of urinary nitrogen was increased. Since fecal nitrogen did not change appreciably the change in urinary nitrogen was reflected directly in the nitrogen balances.

Nitrogen balance data are expressed in terms of metabolic body size ($kg^{\frac{3}{4}}$) but the reader who wishes to convert the data as given to data for a single average rat needs only to divide by three to obtain a useful approximation. Despite statements to the contrary (Rose et al., '54; Leverton et al., '56a,b,c,d,e), protein or amino acid requirements are obviously related in some manner to body size. It is self-evident that the amino acid requirement of the rat, in absolute terms, is less than that of a man, and that the requirement of a man in turn is less than that of a cow. This is not solely a matter of species difference. The adult man, for instance, requires much more protein than the infant. There can be no question about the existence of a relationship between body size and amino acid requirement; the question arises as to how body size is best taken into account. Melnick and Cowgill ('37), Bricker et al. ('45), and Hegsted et al. ('46) demonstrated that nitrogen balance is related to the basal energy metabolism. The synthesis of peptide bonds requires energy and is probably a continuous process and hence the relationship between amino acid requirement and the continuing or basal energy metabolism. There seems to be little or no immediate connection between nitrogen metabolism and the energy metabolism of muscular work. If hard muscular work is continued over long periods, however, the muscles will hypertrophy and in this sense work can lead to nitrogen retention. The basal energy metabolism is most frequently expressed in terms of the classical surface area but the determination of body surface is difficult and uncertain. Brody ('45) and Kleiber

('47) showed that basal heat production is proportional to some power function of the body mass. The three quarters power of the body weight in kilograms serves very well and is a unit which is both easy to determine and easy to use. It will be apparent later that metabolic body size, i.e. $kg^{\frac{3}{4}}$, is a very useful unit when comparing the nitrogen metabolism, as well as the basal energy metabolism, of various species of animals which differ greatly in body weight.

DISCUSSION

The data of table 3 reveal some striking differences among three diets which were isonitrogenous as well as isocaloric and which were fed by stomach tube to the same group of rats. The nitrogen balance on the egg diet (exps. III and V) was significantly better than on the diets which contained the "new" amino acid mixture (exps. II and IV). The nitrogen balance in a half-N period on egg (exp. III), for example, is better than in a preceding full-N period on the "new" amino acid mixture (exp. II). The two egg periods agree rather well. These and certain other relationships are shown graphically in figure 1 where the experiments are represented by a series of lines and are numbered from I to V to correspond with table 1. The two points connected by each line represent the half-N and the full-N periods. K is the slope of the line and is a modification of the nitrogen balance index of ingested nitrogen as proposed by Allison et al. ('46). It is evident that on doubling the intake, the "complete" amino acid mixture (CAA in exp. I) and the proteins of whole egg (exps. III and V) were fully utilized to improve nitrogen balance ($K' > 1.0$). The corresponding increments of the "new" amino acid mixture, however, were only 59 and 64% utilized in experiments II and IV respectively.

In negative and slightly positive nitrogen balance the change in nitrogen balance is a linear function of nitrogen intake (Melnick and Cowgill, '37; Bricker et al., 45; Hegsted et al., '46; Allison et al., '46). It is, therefore, permissible

to extrapolate or interpolate the data represented in figure 1 to obtain an estimate of the total nitrogen required to attain nitrogen equilibrium (NI_e) in each experiment. This computation was made for each rat in 5 experiments and the NI_e values are 166 ± 4, 288 ± 16, 148 ± 3, 203 ± 8, and 134 ± 2 mg/day/kg$^{3/4}$ for experiments I, II, III, IV, and V respectively (table 3). The gradual improvement in nitrogen balance over a period of 25 weeks as shown by the topmost points in experiments I, III, and V in figure 1 is typical of

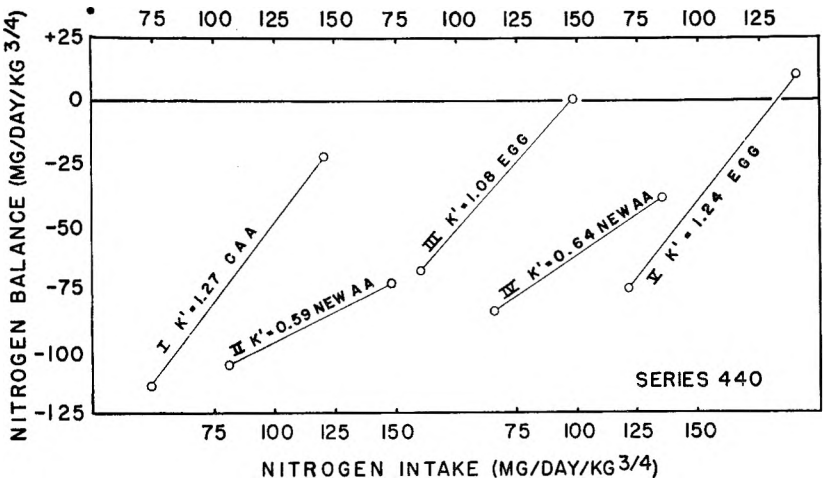

Fig. 1 Nitrogen balance plotted against nitrogen intake from two amino acid mixtures and egg protein.

the adaptation which occurs on a constant intake of a qualitatively adequate source of dietary nitrogen. The "new" amino acid mixture is obviously inferior to either the "complete" amino acid mixture or the proteins of whole egg and the differences are all highly significant ($P < 0.0001$ for all comparisons). It is important to note that the present group of rats (series 440) responded typically to the well standardized dietary regime. In earlier work (Nasset and Anderson, '50) the same amount of egg protein was fed to a different group of rats in experiment III (11th to 15th weeks) and the computed NI_e was 138 which compares well with the

corresponding NI_e of 148 for the present group of animals. The "complete" amino acid mixture has been fed as follows over a period of several years: to 145 rats in experiment I (first to 5th week) to give a mean NI_e of 167 ± 3 mg/day/kg$^{\frac{3}{4}}$; to 11 rats in experiment III (11th to 15th week) to give a mean NI_e of 136 ± 3 mg/day/kg$^{\frac{3}{4}}$; to 40 rats in experiment IV (16th to 20th week) to give a mean NI_e of 136 ± 3 mg/day/kg$^{\frac{3}{4}}$; and to 63 rats in experiment V (20th to 25th week) to give a mean NI_e of 128 ± 2 mg/day/kg$^{\frac{3}{4}}$. On the basis of these data two conclusions seem to be justified: (1) the present series of rats (440) responded in the usual manner, and (2) the adult male rat under these conditions from the 11th to the 25th week makes equally good use of whole egg protein and the "complete" amino acid mixture to maintain nitrogen balance.

The writer sees no point in selecting arbitrarily either a "zone of nitrogen equilibrium," which seems a contradiction in terms, or something indeterminate called "distinctly positive nitrogen balance," on which to base an estimate of amino acid requirements. Nitrogen equilibrium is universally understood to mean the point at which the intake and output of nitrogen are equal and whether this point is precisely attained in actual experimentation is irrelevant. In a properly conducted experiment the point of nitrogen equilibrium is easily computed for each subject and this should remain as a standard reference point. With this in mind the data of Rose et al. ('54) and of Leverton et al. ('56a,b,c,d,e) have been recalculated taking into account, as far as possible, the body size and the point of nitrogen equilibrium. The computed mean minimum essential amino acid requirements for maintenance of nitrogen equilibruim in the adult human male and female as well as the adult male rat are given in table 4.

If metabolic body size is considered it is evident that several of the essential amino acid requirements for these three experimental animals are similar. The only qualitative difference concerns histidine and it seems quite possible that this may not be a real difference. Nasset and Gatewood ('54)

TABLE 4

Computed mean minimum requirements of essential amino acids for maintenance of nitrogen equilibrium in the adult

AMINO ACID	RAT [1]			HUMAN					
	Male			Male [2]			Female [3]		
	mg/day per kg$^{3/4}$	micro-moles	mole [4] ratios	mg/day per kg$^{3/4}$	micro-moles	mole ratios	mg/day per kg$^{3/4}$	micro-moles	mole ratios
Histidine	6.7	43	0.26	0	0	0			
Isoleucine	30	229	1.36	31	236	1.56			
Leucine	16	122	0.73	29	221	1.46	24	183	1.45
Lysine	3.6	25	0.15	25	171	1.13			
Methionine	40	268	1.60	38	255	1.69			
Phenylalanine	18	109	0.65	38	230	1.52	26	157	1.25
Threonine	20	168	1.00	18	151	1.00	15	126	1.00
Tryptophan	4.4	22	0.13	8	39	0.26	7	34	0.27
Valine	18	154	0.92	25	214	1.42	34	290	2.30

[1] From data of Nasset et al., *loc. cit.*

[2] From data of Rose et al., *loc. cit.*

[3] From data of Leverton et al., *loc. cit.*

[4] On the basis of threonine taken as unity.

pointed out that the apparent non-essentiality of histidine for the adult human male as reported by Rose et al. ('51) may be associated with the relatively short feeding periods used by these investigators and the possibility of utilizing endogenous histidine derived from degradation of hemoglobin. The greatest quantitative difference is in the lysine requirement and according to the data in table 4 the human requires about 7 times as much as the rat. The isoleucine, methionine, and threonine requirements for the adult male of the two species could pass for duplicate determinations. The requirement for leucine, phenylalanine, tryptophan, and valine are somewhat higher for man than for the rat. This is to be expected because some of the data for the human male are based on the highest minimum needed to maintain "distinctly positive" nitrogen balance. Four requirements for the human female, as derived from the work of Leverton et al. ('56), lie between those for the human male and the rat, with valine the only exception. These differences may not be significant. If the various experimental techniques and methods of expressing results are taken into account the similarities are striking and they suggest that the essential amino acid requirements per unit of metabolic body size may be the same for the rat and man.

The reasons for the poor showing of the "new" amino acid mixture are unknown. One explanation could be that a single essential amino acid in the presence of a relative excess of the others does not behave the same as when the others are also present in minimal quantities. The sites of protein synthesis apparently require for adequate function the simultaneous presence of all essential amino acids. The probability that one of these sites would be lacking a structural unit at a critical time would seem to be lessened by the presence of a relative excess of even one of the essentials. On the basis of this sort of interaction the "new" amino acid mixture should be improved by supplementation with any one of its 9 component essential amino acids.

Another possibility for the unfavorable showing of the "new" mixture is suggested by the relatively large quantity of glutamic acid (table 2). Amino acid imbalance is now generally recognized as undesirable from several points of view but what consitutes imbalance quantitatively remains to be established. As the result of several years of use the "complete" amino acid mixture (table 2) appears to be nearly equivalent to egg protein in maintaining nitrogen balance in the adult male rat. In this mixture the molar concentration of glutamic acid is 5.60 times as great as that of threonine and in view of the results this probably does not constitute imbalance. In the "new" mixture, however, this same molar ratio is increased nearly 10-fold. It is not unreasonable to assume that this preponderance of glutamic acid may represent imbalance.

The value for NI_e in experiment II with the "new" amino acid mixture is 288. This is 84% greater than would be expected if the "complete" mixture or egg protein had been fed. In making this estimate a uniform rate of change in normal NI_e is assumed. A similar estimate for the second feeding of the "new" mixture (exp. IV) shows that NI_e is 44% greater than normal. These results suggest that nitrogen equilibrium can be obtained with the "new" mixture by feeding less than double the amount actually fed. This brings up the question again as to whether increasing a single amino acid in the mixture will make it equivalent either to the "complete" mixture or to egg protein. In the individual determinations it was found that methionine became limiting with the smallest reduction and lysine required the greatest reduction. The "new" mixture (table 2) represents the following percentage reductions from the amounts found in egg protein: methionine 42, isoleucine 52, threonine 55, tryptophan 69, valine 72, histidine 81, leucine 81, phenylalanine 84 and lysine 94. On the simplest basis of interaction the substitution of more of any one of these 9 amino acids for an isonitrogenous quantity of glutamic acid should improve nitrogen balance. It has been known for some time that the

addition of methionine to a diet very low in nitrogen will improve nitrogen balance (Miller, '44). Similar results for all of the other essentials, except phenylalanine, were reported by Brush et al. ('47). In view of the current interest in amino acid supplementation of diets it is important to determine how much variation in the relative concentration of the essential amino acids may be tolerated before adverse effects become evident.

SUMMARY

An amino acid mixture based on the mean minimum requirements of 9 essential amino acids, determined individually in previous experiments, is significantly inferior to either egg protein or an amino acid mixture which simulates egg protein.

The essential amino acid requirements for the maintenance of nitrogen equilibrium in the rat and man are compared. On the basis of metabolic body size ($kg^{\frac{3}{4}}$), several of the requirements are identical in the two species and several others are closely similar.

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LITERATURE CITED

ALLISON, J. B., R. D. SEELEY, J. H. BROWN AND J. A. ANDERSON 1946 The evaluation of proteins in hypoproteinemic dogs. J. Nutrition, *31:* 237–247.

ANDERSON, J. T., AND E. S. NASSET 1948 Nitrogen balance index and specific dynamic action in rats receiving amino acid mixtures low in isoleucine, methionine or valine. Ibid., *36:* 703–720.

———— 1950 The utilization by the adult rat of amino acid mixtures low in leucine. Ibid., *40:* 625–638.

BRICKER, M., H. H. MITCHELL AND G. M. KINSMAN 1945 The protein requirements of adult human subjects in terms of the protein contained in individual foods and food combinations. Ibid., *30:* 269–283.

BRODY, S. 1945 Bioenergetics and growth with special reference to the efficiency complex in domestic animals. Reinhold Publishing Corp., New York.

BRUSH, M., W. WILLMAN AND P. P. SWANSON 1947 Amino acids in nitrogen metabolism with particular reference to the role of methionine. J. Nutrition, *33:* 389–410.

HEGSTED, D. M., A. G. TSONGAS, D. B. ABBOTT AND F. J. STARE 1946 Protein requirements of adults. J. Lab. Clin. Med., *31:* 261–284.

KLEIBER, M. 1947 Body size and metabolic rate. Physiol. Rev., *27:* 511–541.

LEVERTON, R. M., M. R. GRAM, M. CHALOUPKA, E. BRODOVSKY AND A. MITCHELL 1956a The quantitative amino acid requirements of young women. *I.* Threonine. J. Nutrition, *58:* 59–82.

LEVERTON, R. M., M. R. GRAM, E. BRODOVSKY, M. CHALOUPKA, A. MITCHELL AND N. JOHNSON 1956b The quantitative amino acid requirements of young women. *II.* Valine. Ibid., *58:* 83–94.

LEVERTON, R. M., N. JOHNSON, J. PAZUR AND J. ELLISON 1956c The quantitative amino acid requirements of young women. *III.* Tryptophan. Ibid., *58:* 219–230.

LEVERTON, R. M., N. JOHNSON, J. ELLISON, D. GESCHWENDER AND F. SCHMIDT 1956d The quantitative amino acid requirements of young women. *IV.* Phenylalanine — with and without Tyrosine. Ibid., *58:* 341–354.

LEVERTON, R. M., J. ELLISON, N. JOHNSON, J. PAZUR, F. SCHMIDT AND D. GESCHWENDER 1956e The quantitative amino acid requirements of young women. *V.* Leucine. Ibid., *58:* 355–365.

MELNICK, D., AND G. R. COWGILL 1937 The protein minima for nitrogen equilibrium with different proteins. Ibid., *13:* 401–424.

MILLER, L. L. 1944 The metabolism of DL-methionine and L-cystine in dogs on a very low protein diet. J. Biol. Chem., *152:* 603–611.

NASSET, E. S., AND J. T. ANDERSON 1950 The N balance index of low-valine amino acid mixtures and of whole egg protein in the adult rat. J. Nutrition, *41:* 607–618.

———— 1951 Nitrogen balance index in the adult rat as affected by diets low in L and DL-methionine. Ibid., *44:* 237–248.

NASSET, E. S., J. T. ANDERSON AND A. M. SILICIANO 1951 Nitrogen balance of adult rats fed diets low in L- and DL-threonine. Ibid., *45:* 173–182.

NASSET, E. S., AND M. T. ELY 1952 Nitrogen balance of adult rats fed diets low in L- and DL-lysine, or devoid of arginine. Ibid., *48:* 391–400.

———— 1953 Nitrogen balance of adult rats fed amino acid diets low in L-, DL- and D-tryptophan. Ibid., *51:* 449–462.

NASSET, E. S., AND V. H. GATEWOOD 1954 Nitrogen balance and hemoglobin of adult rats fed amino acid diets low in L- and D-histidine. Ibid., *53:* 163–176.

NASSET, E. S., AND A. M. SILICIANO 1952 Nitrogen balance of adult rats fed diets low in L-, DL- and D-phenylalanine. Ibid., *47:* 437–447.

ROSE, W. C., W. J. HAINES AND D. T. WARNER 1951 The amino acid requirements of man. *III.* The role of isoleucine: Additional evidence concerning histidine. J. Biol. Chem., *193:* 605–612.

ROSE, W. C., G. F. LAMBERT AND M. J. COON 1954 The amino acid requirements of man. *VII.* General procedures: the Tryptophan Requirement. Ibid., *211:* 815–827.

ROSE, W. C., AND R. L. WIXOM 1955 The amino acid requirements of man. *XVI.* The role of the nitrogen intake. Ibid., *217:* 997–1004.

ABSORPTION OF CALCIUM AND STRONTIUM FROM MILK AND NONMILK DIETS[1]

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As a part of an extended investigation on calcium and strontium metabolism it was possible to obtain quantitative data on the effect of milk upon absorption of these two elements in cattle, rats, rabbits and human subjects. With cattle, the usual calcium balance was employed and correlated with the excretion of single oral doses of Ca^{45} and Sr^{89} as criteria of absorption. With the laboratory animals, the retention in bone of orally administered Ca^{45} in milk or as $CaCl_2$ was used to give a measure of absorption and retention (Wasserman et al., '56). The fecal excretion of ingested Sr^{85} by human subjects was used to serve as a measure of absorption under the given test-diet conditions.

A survey of the literature revealed no work in this country on the relative availability of milk versus plant calcium for livestock; however, from work done abroad (Sokolov, '29; Tremazi, '52; Wellmann, '43), there has been an indication of the superiority of milk. From studies with man and rats it appears that milk calcium is generally more available than is plant calcium (Sherman, '47). Salts such as di- and tricalcium phosphate, calcium sulfate, calcium carbonate, calcium lactate, and calcium gluconate have been judged to be

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comparable to milk as a source of calcium (Kempster et al., '40; Patton and Sutton, '52; Pierce et al., '40; Stearns and Jeans, '34); the criteria employed in these studies were primarily skeletal retention of calcium in young growing laboratory animals and net retentions of calcium over relatively long periods of time for man.

The present paper is concerned primarily with the following: (1) the retention of single doses of radiocalcium or radiostrontium as correlated with the usual calcium balance, (2) the direct effect of milk on calcium absorption in different species, and (3) the absorption of radiostrontium in man and cattle as affected by milk.

EXPERIMENTAL

Cattle. Eleven animals raised under ordinary farm management conditions were used. There were two comparable pairs of male Jersey calves, two comparable pairs of male Hereford calves, a 4- and a 7-month-old male Jersey calf, and an 11-year-old nonlactating Jersey cow. Three weeks before the balance trial, one animal from each pair was weaned from the milk diet to one of orchard-grass hay and a commercial concentrate (18 to 20% protein and containing 1% ground limestone and 1% defluorinated rock phosphate). The animals were trained to the metabolism stall and at the start of the 7-day balance period were given orally 2 millicuries of Ca^{45} as the chloride and 0.5 millicuries of Sr^{89} as the chloride. The 4-month and 7-month calves had been weaned to a hay and grain ration, but were returned to a milk diet for this study. After a 7-day balance on the milk diet, they were placed on the same hay and grain ration as the paired calves for a subsequent balance trial.

The aged Jersey cow, in the first trial, received 2 parts orchard-grass hay, 1 part corn, 2 parts soybean oil meal, and enough $CaCO_3$ to provide 30 gm of calcium per day. The ration for the second balance period consisted of 2 parts orchard-grass hay, 1 part corn, 2 parts dry skim milk, and supplied 32 gm of calcium per day.

The total calcium balance was obtained by the usual chemical analysis of feed and feces. The excreta of animals receiving Ca^{45} and Sr^{89} were assayed for radioisotope content by double counting procedures. The methods for handling the animals and analytical techniques were those described by Comar ('55).

Laboratory animals. Albino rats of the Carworth strain were fasted for 24 hours and anesthetized to facilitate passage of the stomach tube. They were divided into three groups: one group received 2 ml of milk containing $Ca^{45}Cl_2$, another group received 2 ml of $Ca^{45}Cl_2$ solution, and the remaining group was allowed to consume 1 gm of a grain mixture [4] followed by a $Ca^{45}Cl_2$ gavage. All dosages contained 5.2 mg of calcium as milk calcium or calcium chloride and 10 microcuries of Ca^{45}.

The same solutions and grain mixture were used for the rabbits; however, the grain was made into a slurry and given by gavage. The 900-gm rabbits (6 weeks old) received either 8 ml of the labeled milk or $Ca^{45}Cl_2$ solutions, which provided 20.8 mg of calcium and 40 microcuries of Ca^{45}. The adult rabbits received a dosage consisting of 39 mg of calcium and 80 microcuries of Ca^{45}.

After dosage, the rats and rabbits were kept for 24 hours without food and then were killed for removal of the femurs. The bones were assayed for Ca^{45} and results were expressed as percentage of dose in both femurs, except with the adult rabbits for which the values represent percentage of dose per femur.

Human subjects. The use of strontium^{85} in human subjects has been described by Spencer et al. ('56) and reviewed by Comar and Wasserman ('56). The present studies were done with chronically ill patients having malignant neoplasms. No milk or milk products were allowed for 24 hours before the administration of the radioisotope. Normal meal schedules were maintained up to and including the last meal at 6 p.m. of the day preceding the experiment. The Sr^{85}

[4] One part wheat germ, one part dried yeast, 4 parts ground corn, 4 parts whole wheat flour.

plus test substance was given at 7 a.m. the next morning. Each subject received one pint of milk containing the $Sr^{85}Cl_2$, or capsules of $CaCl_2$ solution containing the $Sr^{85}Cl_2$. The total calcium supplied was 0.5 gm. No food was allowed for three hours after dosing, and milk or milk products were excluded for another 24 hours.

TABLE 1

Retention of dietary calcium and ingested Ca^{45} and Sr^{89} as affected by diet and age of cattle

DIET	AGE AND BREED	BODY WT.	Ca INTAKE	Ca RETENTION		Ca^{45} RETENTION	Sr^{89} RETENTION	"TRUE" DIGESTIBILITY OF DIETARY Ca
		kg	*gm/day*	*gm/day*	*%*	*%*	*%*	*%*
Milk	2-day, J.[1]	32.7	8.0	8.0	100	99	95	100
Milk	2-day, J.	30.9	8.0	7.9	98	98	93	100
Milk	2-month, H.[1]	79.0	9.1	6.9	76	94	86	89
Hay and grain	2-month, H.	60.8	1.7	−0.7	..	77	69	12
Milk	3-month, J.	81.7	11.9	..	..	96	86	..
Hay and grain	3-month, J.	81.7	16.1	..	..	58	47	..
Milk	4-month, J.[2]	103	13.1	10.4	80	..	..	91
Hay and grain	4½-month [1,2]		5.9	0.28	4.8	..	..	33
Milk	5-month, H.	132	11.4	6.2	54	79	64	72
Hay and grain	5-month, H.	144	31.9	3.4	11	15	13	18
Milk	7-month, J.[2]	168	18.3	13.1	72	..	..	84
Hay and grain	7½-month [2]		7.2	0.86	12	..	..	47
Skim milk	11-yr., J.[2]	400	32	−1.7	5.2	..	..	23
Grain	11-yr., J.[2]		30	−6.6	−22	..	..	8

[1] Jersey (J.), Hereford (H.).

[2] Same animal used for both rations.

Urinary and fecal collections were made for 4 to 8 days, during which time the major part, if not all of the unabsorbed Sr^{85} had been eliminated as shown by the low fecal levels. Before the second test, the excreta were assayed to make sure that the levels were not high enough to interfere with the subsequent measurements.

EXPERIMENTAL RESULTS

Cattle. The over-all results are summarized in table 1. The "true" digestibility values were calculated from the

balance data by estimation of the fecal endogenous loss, which is essentially independent of short time dietary changes (Hansard et al., '54; Comar, '56). It may first be noted that the "true" digestibility of the calcium of the milk diet was always definitely superior to that of the nonmilk diet even in the aged animal. From the standpoint of age, the digestibility on milk decreased from 100% at two days to 72 to 84% at 5 to 7 months. No trends were apparent on the effect of age on digestibility of the hay and grain ration, probably because of the variability in the calcium intake.

The radioisotope values support the usual balance data and, in addition, provide information on one pair of animals for which chemical values were not available. It is important to note that the retentions of Ca^{45} and Sr^{89}, with one exception, gave essentially similar results as the conventional digestibility trials. As expected, the Ca^{45} retention was always slightly higher than that of Sr^{89} (Comar et al., '56).

The superiority of the milk ration is emphasized by consideration of retention in terms of comparative intakes of calcium. The two-month-old calf on hay and grain received only 1.7 gm of calcium per day and even at this low intake, the efficiency of absorption did not approach that of the animal on milk; the same situation occurred in the 4-month and 7-month-old calves. It is interesting to note that in the 5-month calves, the animal on hay and grain was able to retain 3.4 gm of calcium from an intake of 31.9 gm, whereas on milk, the animal retained 6.2 gm or almost twice as much from an intake of only 11.4 gm.

Table 2 presents the results of an additional experiment designed to demonstrate the reversibility of the effect. A one-month-old Jersey calf was weaned to hay and grain. Since a calcium balance had not been run before weaning, it was estimated from similar animals that the "true" digestibility would be about 90%. After the animal had been on hay and grain for 30 days, the calcium balance and Ca^{45} retention were determined. After the balance trial, the calf

was returned to the milk diet for 7 days and the calcium balance and Ca^{45} retention were measured again.

It may be first noted that the values are in general agreement with those of table 1. There seems little question that the animal did absorb milk calcium efficiently even though it had been on the hay and grain diet for 30 days previously. Again, of particular importance is the fact that the calf was able to retain 8.8 gm of calcium daily from the milk ration as compared with only 3.9 gm from the hay and grain.

Rats and rabbits. Table 3 summarizes the data for rats and rabbits. With both the younger and older rats, there

TABLE 2

Reversibility of effects of hay and grain on retention of dietary calcium in a Jersey calf

DIET	AGE	BODY WT.	DAILY Ca INTAKE	DAILY Ca RETENTION		Ca^{45} RETENTION	"TRUE" DIGESTIBILITY OF DIETARY Ca
	mo.	*kg*	*gm.*	*gm*	%	%	%
Milk	1	...	...	...	...	...	90 [1]
Hay and grain	2	61.7	12.8	3.9	29.4	41.7	37.5
Milk	2½	65.8	10.7	8.8	82.4	90.1	91.6

[1] Estimation from values obtained for similar animals.

was an increased absorption of $calcium^{45}$ from milk over nonmilk sources that was significant at the 1% level. The milk increased the absorption by about 1.5 times. There were no statistical differences between the groups receiving Ca^{45} as the chloride alone or in the presence of grain.

Studies with young rabbits weighing about 920 gm indicated that there was no effect of milk on promoting Ca^{45} absorption. Further investigations showed that these animals are capable of absorbing close to 100% of the Ca^{45} even on a nonmilk diet. Thus, the enhancement of calcium absorption by a dietary addition could not be evaluated in the young rabbit. Since older animals generally have a lower absorption, the studies were repeated with mature rabbits. As indicated in table 3, there were no significant differences with treatment.

TABLE 3

Effect of milk on absorption of calcium by rats and rabbits

SPECIES	AV. WT.	TREATMENT	NO. OF ANIMALS	Ca^{45} IN FEMURS	RELATIVE AVAILABILITY ($CaCl_2$ = 100)
	gm			*% of dose*	*%*
Rat	113	$CaCl_2$	6	4.8 ± 0.5 [1]	100
		$CaCl_2$ + grain	6	5.5 ± 0.2	114
		Milk	7	7.2 ± 0.3	150
	304	$CaCl_2$	6	2.1 ± 0.1	100
		$CaCl_2$ + grain	5	2.0 ± 0.4	95
		Milk	6	3.1 ± 0.2	148
Rabbit	920	$CaCl_2$	6	9.0 ± 0.5	100
		$CaCl_2$ + grain	5	8.4 ± 0.4	93
		Milk	6	8.8 ± 0.3	98
	3760	$CaCl_2$	4	1.7 ± 0.2	100
		Milk	5	1.2 ± 0.3	71

[1] Mean ± standard error of the mean; values are percentage of dose in both femurs except for adult rabbits, the values for which are percentage of dose in one femur.

TABLE 4

Absorption and retention of Sr^{85} by human subjects as affected by milk

Subject	A	B	C	D
Age, *years*	64	68	58	15
Sex	M	M	M	F
Condition	generalized lymphosarcoma	lymphoepithelioma of nasopharynx	generalized lymphosarcoma	medulloblastoma of cerebellum
Dose, *μc per test*	20	20	20	5
% absorbed				
$CaCl_2$	14	45	41	16
Milk	11	65	99	83
% retained				
$CaCl_2$	13	37	33	5.1
Milk	10	64	95	75
% of dose in urine				
$CaCl_2$	1.8	7.7	7.8	11.3
Milk	0.7	0.5	3.6	7.8
% of absorbed dose in urine				
$CaCl_2$	13	17	19	69
Milk	5.5	0.8	3.7	10

Human subjects. The data on human subjects are summarized in table 4. It is recognized that there were many variables such as age and physical condition. Nevertheless it did appear that absorption and retention were significantly increased (at the 10% level) when the Sr^{85} was given with milk. In one patient out of 4, there was no effect due to milk. In all patients, the milk apparently caused an increased retention of absorbed Sr^{85} as judged by the values for percentage of absorbed dose excreted in the urine.

DISCUSSION

The data in tables 1 and 2 show that for cattle there is good correlation between the usual calcium balance and the retention of single ingested doses of radiocalcium or radiostrontium. In 7 out of 8 trials, the results from Ca^{45} retention and calcium balance agreed within 10%. The one deviation probably represented experimental errors because of the very small calcium intake. This means that the single dose of Ca^{45} as $CaCl_2$ behaved in the same way as did the calcium of the diet (whether in milk, or hay and grain) during the balance period.

Similarly, in 8 possible comparisons, the Sr^{89} values were lower but averaged within 11% of the Ca^{45} values. In rats and man, however, the strontium retention would be expected to be much lower because of greater absorptive discrimination between calcium and strontium (Comar et al, '56; Harrison et al., '55). Nevertheless, Wasserman et al ('56) and Comar et al ('56) have shown that with rats the factors in milk enhancing calcium absorption also increase strontium absorption but to a somewhat greater degree.

There seems little question that the cattle were able to absorb and retain two to three times more calcium from the milk than from a nonmilk ration. This may be of practical importance on account of the trend toward reduction of costs by feeding milk replacements rather than fresh milk to dairy calves. Also, there is an interest in raising the

percentages of roughage in calf rations. The present results indicate that a previously reported difference in calcium absorption between one-month and 6-month-old calves was caused more by difference in ration than by difference in age (Hansard et al., '54).

Rats apparently respond to milk in the same way as cattle. In another study based on daily feeding of Ca^{45} and Sr^{89}, rats on a milk diet absorbed almost twice as much Ca^{45} but almost 4 times as much Sr^{89} as rats on commercial pellets (Comar et al., '56). This supports the data in table 3 showing the favorable effect of milk in the rat but emphasizes the need for caution in the interpretation of strontium retention values in relation to calcium metabolism. In regard to other animal species, it has been shown that rabbits did not respond to milk; results to be published elsewhere also show that milk did not increase calcium absorption by chicks.

The data on human subjects are primarily of interest in providing information on the absorption and retention of radiostrontium in man. This is of particular importance in assessment of the potential hazard of contamination of the biosphere with radiostrontium. Comar and Wasserman ('56) have reviewed such data in the literature and have reported 9 values (for absorption of strontium in man) which averaged about 30% and ranged from 14 to 42%. This is in general agreement with the values reported for the $CaCl_2$ test. There seems little question that the milk increased both the absorption and retention of the Sr^{85}.

It is difficult to estimate the calcium absorption from the observed Sr^{85} absorption in man. This is because normally, the absorption of calcium in man would be expected to be about twice that of strontium (Harrison et al., '55); yet milk would probably increase strontium absorption more than it would increase calcium absorption. In any event, it seems probable that milk must have increased calcium absorption and that such absorption must have been very efficient, approaching 90 to 100%. In contrast, published values obtained by classical

methods indicate that milk calcium is only from 19 to 30% utilizable by children and adults (Kempster et al., '40; Kinsman et al., '39; Schroeder et al., '46; Steggerda and Mitchell, '41). A possible explanation is that in the conventional balance study, the calcium source to be tested is incorporated into a well-balanced diet, and the results may express the availability of the dietary calcium as a whole rather than for the particular supplement. Also, the usual balance results have not included the contribution of the fecal endogenous losses and thus underestimate the "true" digestibility by appreciable amounts. For example, Laszlo et al. ('56) report a subject with a daily intake of 529 mg and fecal excretion of 408 mg of calcium to give an apparent digestibility of 23%. The endogenous fecal calcium, however, was 91 mg/day so that the "true" digestibility was 41%.

The type of procedure employed in the present paper eliminates many of the difficulties of the determination of chemical balance in addition to being much less laborious. The endogenous factor is not important because only a small proportion of the radioisotope entering the body is excreted into the gut during the experimental period (Comar et al., '53). The absorption of a mineral from a particular supplement or salt can be determined without having the subject on an unbalanced diet. Although there are no significant radiation problems with animals, this is an important obstacle to studies with man. It is hoped that the short-lived Ca^{47} will be available eventually in order to extend these methods to fundamental and diagnostic applications.

It is of interest to consider the possible reasons for the enhancement of calcium absorption by milk. The ready reversibility of the effect as demonstrated in table 2 would indicate that there is a direct-acting causative agent in the diet, rather than some deep-seated physiological change in the animal brought about by the diet. With ruminants, it is necessary to consider the possibility of an inhibition of absorption by oxalates or phytates of the hay and grain ration; however, this does not seem likely because such substances are generally

destroyed before reaching the site of most active calcium absorption (Talapatra et al., '48; Reid et al., '47). It is also possible to rule out vitamin D differences between the milk and nonmilk treatments. The fat portion of milk can be eliminated as a factor since the response was observed when fat-free milk solids were used.

Of the milk constituents, lactose has been shown to increase calcium absorption in calves (Robinson et al., '29), and in rats (Wasserman et al., '56). Also, the amino acids, lysine and arginine, which are present in large amounts in milk protein, have shown a marked ability to increase calcium absorption in rats (Wasserman et al., '56). At this time it is difficult to assess the relative importance of lactose or the amino acids of milk protein in the enhancement of calcium absorption.

SUMMARY

1. The availability of calcium from milk was contrasted with that from $CaCl_2$ in rats, rabbits and human subjects, and with calcium supplied with grain in cattle, rats and rabbits.

2. In cattle, the retention of single oral doses of Ca^{45} and Sr^{89} gave essentially the same results as the conventional determination of calcium balance.

3. Calves on a milk diet absorbed and retained a very high percentage of the calcium present. Similar animals on a hay diet and grain diet showed a much lower absorption and retention. A calf on hay and grain for 30 days showed the typical high calcium utilization when returned to a milk diet. The calcium retention of an 11-year-old cow was improved by the addition of dried skim milk in the ration.

4. Young and old rats absorbed about one and a half times as much calcium^{45} from milk as from a solution of $CaCl_2$ or from $CaCl_2$ + grain.

5. Young and old rabbits showed no increased Ca^{45} absorption from milk.

6. Three out of 4 human subjects absorbed an average of 34% ingested Sr^{85} from $CaCl_2$ as compared with 82% from milk.

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LITERATURE CITED

COMAR, C. L. 1955 Radioisotopes in Biology and Agriculture — Principles and Practice, McGraw-Hill Book Co., Inc., New York.

———— 1956 Radioisotopes in animal physiology and nutrition — mineral metabolism. Proc. Int. Conf. on Peaceful Uses of Atomic Energy, *12:* 245–251.

COMAR, C. L., R. A. MONROE, W. J. VISEK AND S. L. HANSARD 1953 Comparison of two isotope methods for determination of endogenous fecal calcium. J. Nutrition, *50:* 459–467.

COMAR, C. L., AND R. H. WASSERMAN 1956 Radioisotopes in the study of mineral metabolism. Progress in Nuclear Energy, Biological Sciences, Pergamon Press, London Series *VI:* 153–196.

COMAR, C. L., R. H. WASSERMAN AND M. M. NOLD 1956 Strontium-calcium discrimination factors in the rat. Proc. Soc. Exp. Biol. Med., *92:* 859–863.

HANSARD, S. L., C. L. COMAR AND M. P. PLUMLEE 1954 The effects of age upon calcium utilization and maintenance requirements in the bovine. J. Animal Sci., *13:* 25–36.

HARRISON, G. E., W. H. A. RAYMOND AND H. C. TRETHEWAY 1955 The metabolism of strontium in man. Clinical Science, *14:* 681–695.

KEMPSTER, E., H. BREITER, R. MILLS, B. M. MCKEY, M. BERNDS AND J. OUTHOUSE 1940 The utilization of the calcium of di-calcium phosphate by children. J. Nutrition, *20:* 279–287.

KINSMAN, G., D. SHELDON, E. JENSEN, M. BERNDS, J. OUTHOUSE AND H. H. MITCHELL 1939 The utilization of the calcium of milk by pre-school children. Ibid., *17:* 429–441.

LASZLO, D., H. SPENCER, M. BROTHERS, E. BERGER AND H. E. HART 1956 Biological studies on calcium, strontium, lanthanum and yttrium. Proc. Int. Conf. on Peaceful Uses of Atomic Energy, *10:* 62–67.

PATTON, M. B., AND T. S. SUTTON 1952 The utilization of calcium from lactate, gluconate, sulfate, and carbonate salts by young college women. J. Nutrition, *48:* 443–452.

PIERCE, H. B., R. G. DAGGS, A. B. MESERVEY AND W. J. SIMEOX 1940 The retention of calcium and phosphorus by pre-school children. Ibid., *19:* 401–414.

REID, R. L., M. C. FRANKLIN AND E. G. HALLSWORTH 1947 The utilization of phytate phosphorus by sheep. Australian Vet. J., *23:* 136–140.

ROBINSON, C. S., C. F. HUFFMAN AND M. F. MASON 1929 The results of the ingestion of certain calcium salts and of lactose. J. Biol. Chem., *84:* 257–267.

SCHROEDER, L. J., W. M. CAHILL AND A. H. SMITH 1946 The utilization of calcium in soybean products and other calcium sources. J. Nutrition, *32:* 413–422.

SHERMAN, H. C. 1947 Calcium and phosphorus in foods in nutrition. Columbia University Press, New York, p. 93.

SOKOLOV, E. A. 1929 Metabolism of nitrogen and calcium in calves during the period of milk feeding. Nauch-Agron. Zhur., *6:* 429–437 (as quoted in C. A. 1930, *24:* 3536).

SPENCER, H., M. BROTHERS, E. BERGER, H. E. HART AND D. LASZLO 1956 Strontium^{85} metabolism in man and effect of calcium on strontium excretion. Proc. Soc. Exp. Biol. Med., *91:* 155–157.

STEARNS, G., AND P. C. JEANS 1934 Utilization of calcium salts by children. Ibid., *32:* 428–430.

STEGGERDA, F. R., AND H. H. MITCHELL 1941 Further experiments on the calcium requirement of adult man and the utilization of the calcium in milk. J. Nutrition, *21:* 577–588.

TALAPATRA, S. K., S. C. RAY AND K. C. SEN 1948 Calcium assimilation in ruminants on oxalate-rich diet. J. Agr. Sci., *38:* 163–173 (C. A. 1950 *44:* 9524B).

TREMAZI, I. A. 1952 Mineral metabolism in growing calves. Pakistan J. Sci. Res., *4:* 45–50.

WASSERMAN, R. H., C. L. COMAR AND M. M. NOLD 1956 The influence of amino acids and other organic compounds on the gastro-intestinal absorption of calcium^{45} and strontium^{89} in the rat. J. Nutrition, *59:* 371.

WELLMANN, D. 1943 Changes in the metabolism of the organism in case of milk (meat), mixed, and vegetable diets. Math. naturw. Anz. ungar. Akad. Wiss., *62:* 186–200 (as quoted in C. A. 1947 *41:* 7464B).

NUTRITION OF RATS WITH COMPOUNDS OF KNOWN CHEMICAL STRUCTURE[1]

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Many unsuccesful attempts have been made during the last 70 years to maintain animals with rations containing compounds of known chemical structure. Such studies have contributed to the discovery and recognition of the nutritional significance of vitamins, amino acids and other dietary essentials. The knowledge thus gained through the earlier work by many others, the accomplishments of the organic chemists and the current availability of the necessary compounds in sufficient quantities have made the present studies possible.

The experiments summarized in this paper were based on the demonstration (Schultze, '56) that rats could be maintained, without evidence of progressive nutritional impairment, for as long as 4 successive filial generations with protein-free amino acid rations in which 3% of a lipid component was the only crude natural product. By substituting a pure source of linoleic acid for the natural lipid component of one of the amino acid rations previously used, it became possible to feed to three successive generations of a mammallan species a ration of which all ingredients are compounds of known chemical structure.

This work has an important bearing on various reports concerning the necessity, for the survival of the young rat, of

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an animal protein factor (Piccioni et al., '51) of orotic acid (Moruzzi et al., '56), of squalene (El Ridi et al., '55) and of the unrecognized component in corn oil or wheat germ oil, the existence of which Keane et al. ('51) proposed but Dryden et al. ('56) could not confirm. The design of the present experiments also afforded a comparison of an amino acid ration containing natural fats with a similar ration in which the methyl linoleate-urea inclusion complex (Holman and Ener, '54) furnished the sole source of fatty acids.

When these studies were nearing completion, two brief reports appeared in which Greenstein and his associates (Greenstein et al., '56; Birnbaum et al., '56), described the formulation of a "soluble diet for rats composed of chemically defined substituents." They reported that "the animals were capable of breeding and subsequent lactation."

EXPERIMENTAL

Rations. The rations were patterned after ration AA_{16} (Hallanger and Schultze, '56) which contained sucrose, 18.3% of a mixture of 16 amino acids, 14 vitamins, choline chloride, 10 inorganic salts, 2% of wheat germ oil and 1% of corn oil. Details of their composition are given in table 1. In ration AA_{20} which was assembled from compounds of known chemical structure the lipid components were furnished by 1.25% of the crystalline urea-methyl linoleate inclusion complex and 1% of a suspension of vitamin A acetate, vitamin D_3 and α-tocopherol in redistilled propylene glycol. The latter compound, found by Guerrant et al. ('47) to be without harmful effect on growth and reproduction of rats in concentrations far exceeding those used in this study, had also the advantage of eliminating the dustiness of the ration To ration AA_{21} which contained 3% of natural fat, urea and propylene glycol were added in the amounts contained in ration AA_{20}. The amino acids were crystalline commercial compounds; no attempt was made to purify them; the isoleucine was stated by the manufacturer to contain L-isoleucine

50% and D-alloisoleucine 50%. The rations were mixed at least once a week, stored at 4°C. and fed ad libitum.

Animals. The strain of rats used and their management were the same as previously described (Schultze, '56). Among the parent (P) generation some rats (groups 1 and 5, table 2) were fed the rations described herein, after they had successfully raised two litters on a protein-free amino acid ration (Schultze, '56, table 3, group 9). Others (groups 2 and 6, table 2), whose dams consumed a ration containing

TABLE 1

Composition of rations

COMPONENT	RATION NO.	
	AA_{20}	AA_{21}
	gm	*gm*
Amino acid mixture II [1]	183.40	183.40
Sucrose + vitamin mixture [2]	20.00	20.00
Salt mixture [3]	40.00	40.00
Propylene glycol + vitamin mixture [4]	10.00	....
Propylene glycol, distilled	....	9.90
Methyl linoleate-urea complex [5]	12.50	....
Urea	....	10.00
Sucrose	734.10	706.30
Wheat germ oil	....	20.00
Corn oil + vitamins A and D_3 [6]	....	10.00

[1] L-Arginine monohydrochloride 7.65; L-histidine monohydrochloride 5.21; DL-isoleucine 19.94; L-leucine 18.97; L-lysine hydrochloride (95%) 11.06; DL-methionine 5.40; DL-phenylalanine 8.00; DL-threonine 12.00; DL-tryptophan 2.78; DL-valine 21.60; DL-aspartic acid 9.72; DL-alanine 8.66; L-cystine 5.01; L-glutamic acid 36.74; L-tyrosine 9.90; glycine 0.76 gm.

[2] Twenty grams of the mixture contain 18.455 gm sucrose + 5 mg thiamine chloride; 10 mg riboflavin; 5 mg pyridoxine hydrochloride; 50 mg calcium pantothenate; 20 mg nicotinic acid; 400 mg i-inositol; 1000 mg choline chloride; 0.2 mg folic acid; 10 mg para-amino benzoic acid; 0.2 mg biotin; 40 mg D-mannitol containing 0.04 mg vitamin B_{12}; 5 mg 2-methyl-1,4-naphthoquinone.

[3] Contains $CaCO_3$, 12.150; K_2HPO_4, 12.900; $CaHPO_4$ (medicinal powder), 3.000; NaCl, 6.700; $MgSO_4 \cdot 7H_2O$, 4.080; ferric citrate, 1.100; $MnSO_4$, 0.020; $CuSO_4 \cdot 5H_2O$, 0.012; ZnO, 0.010; KI, 0.032 gm.

[4] Ten grams of the mixture contain 3.34 mg vitamin A acetate, 0.0375 mg vitamin D_3, 100 mg dl-α tocopherol, 9.897 gm propylene glycol.

[5] Purchased from The Hormel Foundation, Austin, Minnesota. Prepared from methyl linoleate (over 99.5% purity). Ratio urea : methyl linoleate 4 : 1 w/w.

[6] Ten grams of mixture contain 3.34 mg vitamin A acetate, 0.0375 mg vitamin D_3.

mainly rolled oats and casein (Hallanger and Schultze, '56), were started on rations AA_{20} and AA_{21} when they were 21 days old. The offspring (F_1 generation) of group 2 were fed the same respective rations until they had reared two litters and some animals from the latter (F_2 generation) were again raised to maturity and produced one litter. For reasons of economy and because of previous experience with essentially the same ration (Schultze, '56) the experiments with ration AA_{21} were not extended through the F_2 generation. Litter mates were used for all comparisons of the two rations shown in table 1. The young were weaned at 28 days of age. The mothers were permitted to rear all of the young born. After weaning of the second litter the lipid content of the livers of the mothers in the P and F_1 generation was determined by the method of Hedin and Schultze ('55).

RESULTS

Post-weaning weight gains. As shown in table 2, the rats of groups 2, 3, 4 made significantly ($P < 0.01$) smaller weight gains than those of groups 6 and 7, whose growth rate was about the same as previously observed with very similar rations (Schultze, '56). With ration AA_{20} there developed, with successive generations, a gradual decline of the growth rate which was statistically significant ($P < 0.01$) only when the P and F_2 generations were compared. The addition of 1 mg of dl-6-thioctic acid per kilogram of ration AA_{20} did not improve the growth rate. The subnormal growth rate of the rats fed rations AA_{20} or AA_{21} may be due to the presence of D-amino acids in the ration (Wretlind, '56; Phillips and Berg, '54) or to an imbalance among individual amino acids (Elvehjem, '56; Harper, '56). But it must also be reconciled with the fact that under the stress of lactation many of the same rats produced sufficient milk from the same dietary com ponents to enable their litters to attain in three weeks a weight increment of 180 to 200 gm. A change in the endocrine balance may have stimulated greater food consumption during pregnancy and lactation.

TABLE 2

Growth and reproductive performance of rats

ITEMS COMPARED	LIPID COMPONENT OF RATION											
	Methyl-linoleate-urea						Vegetable oils					
	Ration AA_{20}						Ration AA_{21}					
Group no.	1	2		3		4	5	6		7		
Generation	P^1	P^2		F_1		F_2	P^1	P^2		F_1		
Weight gain, 6 weeks growth period, gm	..	86.3		83.0		79.6	..	93.5		101.0		
Number of animals bred	9	15	15	21	21[3]	16	6	10	10	11	11[4]	
Pregnancy	3rd	1st	2nd	1st	2nd	1st	3rd	1st	2nd	1st	2nd	
Weight gain during pregnancy, gm	67.2	65.3	58.6	51.3	49.9	61.9	76.2	79.5	71.3	68.3	68.8	
Number of litters born	9	15	15	21	19	16[5]	6	10	10	11	10	
Mean number of yonng born per litter	8.0	7.4	5.7	5.5	5.6	6.5	9.0	8.6	9.2	8.2	7.6	
Mean number of young per litter weaned	6.4	7.1	5.5	6.2	5.8	6.3	7.7	7.9	9.0	8.1	7.3	
Mortality of young in 28 days, %	12.1	10.8	11.7	4.5	7.5	10.2	14.8	8.1	16.1	2.2	4.0	
Mean weight of young, 28 days old, gm	36.0	38.6	37.1	34.1	36.5	37.2	43.2	41.8	37.8	42.6	39.1	
Mean weight change of mothers 7 days	−4.8	−5.2	−3.2	−3.2	−0.3	−3.9	−5.2	−7.8	−0.3	−6.0	+1.2	
during lactation, gm 28 days	−0.8	−6.2	−12.8	−5.1	−3.9	−4.1	−8.0	−4.8	−3.0	−2.5	−0.1	

[1] These animals were fed a protein-free amino acid ration containing 3% of vegetable oil before the third pregnancy.

[2] These animals were fed the rations AA_{20} or AA_{21} since weaning at 21 days of age.

[3] Two animals failed to conceive.

[4] One animal failed to conceive.

[5] Including one abortion.

Weight at maturity. The rats fed ration AA_{20} had a smaller weight after weaning of the second litters when they were from 28 to 32 weeks old. At that time those of groups 2 and 3 had mean weights of 188 ± 4.7 [2] gm and 187 ± 2.9 gm, respectively, while their litter mates in groups 6 and 7 had attained weights of 219 ± 3.31 and 207 ± 4.05 gm, respectively. This significant difference ($P < 0.01$) is not accounted for by differences in loss of weight during the second lactation.

Reproductive performance with ration AA_{20}. The data for groups 1 to 4, table 2, demonstrate that it is possible to maintain a mammalian species, without indications of serious progressive deterioration, for at least three successive generations with a ration assembled from compounds of known chemical structure. Among 100 pregnancies there was only one abortion and two terminated apparently in resorption of the fetuses. The mean number of young born per litter, in the second litter of the P generation and in subsequent generations, was relatively low. It is not known whether this reflects impairment of ovulation, of implantation or prenatal deaths of some fetuses. The weight gains of the mothers during pregnancy were subnormal although the mean weight of the new born young was normal. The preweaning mortality of the young was low and did not increase with successive generations. Lactation, as reflected by the preweaning weight increments of the young, was distinctly subnormal but the mothers did not incur a severe weight loss during lactation.

There was no evidence that prolonged maintenance of rats on ration AA_{20} through several generations would eventually lead to reproductive failure or impairments other than those pointed out above. Throughout this experiment the rats fed ration AA_{20}, although somewhat smaller than their litter mates fed ration AA_{21}, appeared to be in excellent condition; their coats were smooth and sleek. The reproductive capacity of male rats was not extensively investigated but several females were successfully bred by F_1 generation males.

[2] Standard error of the mean.

The effect of natural fat. The data for groups 5 to 7, table 2, show that the presence of 2% of wheat germ oil and 1% of corn oil in ration AA_{21} improved reproduction and lactation. Statistical comparisons between groups 5 to 7 and groups 1 to 3 showed that the weight gains during pregnancy were significantly greater ($P < 0.01$) in both pregnancies of groups 6 and 7. This reflects in part the larger number of young born per litter in the groups fed the vegetable oils. The difference in the number of young born per litter was statistically significant in the first litters of group 7 ($P = < 0.05, > 0.02$) and in the second litters of group 6 ($P = < 0.01$) as well as in the mean of all litters born to

TABLE 3

Effect of lactation on liver

RATION AND NO. OF YOUNG NURSED PER LITTER	NO. OF RATS	LIVER		
		% of body weight	Dry weight, % of fresh	Lipids, % of dry weight
AA_{20}: 0–2	9	4.32 ± 0.06 [1]	29.1 ± 0.83 [1]	24.8 ± 1.3 [1]
AA_{21}: 2	1	4.33	27.3	22.8
AA_{20}: more than 3	32	5.94 ± 0.21	33.4 ± 1.04	43.6 ± 2.30
AA_{21}: more than 3	24	6.76 ± 0.25	38.0 ± 1.15	54.9 ± 2.78

[1] Standard error of the mean.

mothers fed ration AA_{21} ($P = < 0.01$). Lactation, as reflected by the 28-day weight of the young, was improved in all groups. The difference in this criterion was statistically significant ($P = < 0.01$) in all comparisons between young of groups of litter mates except in the second litters of the P generation; but in this case too, ration AA_{21} supported much better lactation because a mean of 9.0 young per litter attained a greater weight than a mean of 5.5 young raised by litter mates fed ration AA_{20}. The weight changes of the mothers in groups 5 to 7 were not significantly different from those in groups 1 to 3, either 7 or 28 days post partum.

Fatty livers during lactation. Among the rats of groups 1 to 3, table 2, those which were subjected to only a slight stress of lactation because they lost their litters or raised

only 1 to 2 young had normal livers as shown in table 3. In contrast, rats which raised three or more young per litter developed hypertrophied and fatty livers. In this respect the rats of groups 5 to 7, fed ration AA_{21}, were more severely affected than those fed ration AA20, with significant differences in the liver size as percentage of body weight ($P = 0.02$), the dry weight of the liver ($P < 0.01$), and the lipid content ($P < 0.01$). From the available evidence no decision can be reached whether these differences are due to the presence of the natural fats in ration AA_{21} or to the greater stress of lactation caused by the larger number of young per litter. The development of hypertrophied and fatty livers in rats fed amino acid rations and subjected to the stress of lactation is in accord with earlier observations (Hallanger and Schultze, '56).

DISCUSSION

On the basis of available evidence it cannot be decided whether the improved performance of rats fed ration AA_{21} is due to the presence of fat *per se*, to a higher content of unsaturated fatty acids or to some other specific component of the lipids. Unpalatability of ration AA_{20} does not appear to be a major factor in this respect because during pregnancy and lactation the consumption of this ration increased greatly. Loosli et al. ('44) observed that the addition of 5.5% of corn oil to a fat-low ration improved lactation of rats to an extent not accomplished by amounts of ethyl linoleate considered to be sufficient to meet the requirements of the rat. Deuel and Reiser ('55) have recently reviewed various aspects of the nutritional significance of unsaturated fatty acids. Mackenzie et al. ('39) could find no evidence for the existence of a hitherto unknown fat-soluble factor for growth and reproduction of the rat. Regardless of the improved performance of the rats fed ration AA_{21} the results summarized in table 2 provide no evidence for the essential nature of squalene or of other unrecognized dietary components for the survival of the young rat or of the species for several generations.

The results of these experiments should not be interpreted to indicate that the nutritional requirements for the life of a mammalian species are now completely understood, even qualitatively. Thus it is not known to what extent microbial synthesis in the intestine or coprophagy contributed to the nutrition of these animals. Necropsy usually revealed the presence of feces in the stomach even though the animals were kept in cages with raised screens except shortly before parturition and during lactation.

Ration AA_{20} contained large amounts of compounds isolated from natural products. Although crystalline and purified commercially, such components as sucrose, the L-amino acids (except lysine) and the inorganic salts may carry small amounts of natural impurities which are important for the nutrition of the rat. Until the absolute purity of all components of a ration has been rigorously established, the admonition and prediction [3] which Dumas (1871) made 85 years ago applies with equal force today.

It must also be recognized that failure to demonstrate the necessity of a certain dietary component with artificial rations of the type employed in this study does not necessarily imply that the same component would be equally dispensible in rations compounded from crude or partially purified natural products which may be unbalanced with respect to some dietary essentials. A striking example of the serious effects of nutritional imbalance and its correction is furnished by the recent observations on parakeratosis of swine (Lewis et al., '56). While no evidence could be found in this study for the requirement for factor 3 (Schwartz, '54), for orotic acid (Moruzzi et al., '56) or for squalene (El Ridi et al., '55), the rations used by these investigators contained crude products to varying extent. The nutritional unavailability of

[3] "Il est donc toujours prudent de s'abstenir de prononcer sur l'identité de ces mélanges indéfinis employés a l'entretien de la vie, où les moindres traces de matière et les plus insignificantes peuvent se montrer non-seulement efficacis, mais encore indispensables. A mesure que la science étend son domaine, on est même sûr de voir se multiplier les démonstrations de l'opportunité de cette réserve."

some components of natural products or the presence therein of anti-metabolites or antagonists could lead to nutritional failure with rations which, on the basis of chemical analyses for dietary essentials should be adequate. Thus, there is no satisfactory explanation available for the very high incidence of mortality frequently observed in this laboratory (Gander and Schultze, '55) among young rats fed rations composed of cereal grains, supplemented with leached casein and with the same mixture of vitamins and salts as employed in this study.

SUMMARY

1. Rats have been maintained for three successive generations with a ration assembled from compounds of known chemical structure. There was no evidence of progressive nutritional failure during successive generations.

2. The incidence of mortality of the young was very low but pre-weaning and post-weaning weight gains and the weight attained at maturity were subnormal.

3. The addition of 3% of natural fat improved the ration with respect to litter size, pre-weaning and post-weaning weight gains of the young and the weight attained after weaning of the second litter.

4. The significance and pratical limitations of these observations have been discussed.

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LITERATURE CITED

BIRNBAUM, S. M., M. WINITZ, R. H. BROOKS AND J. P. GREENSTEIN 1956 A water-soluble synthetic diet. Federation Proc., *15:* 221.

DEUEL, H. J. JR., AND R. REISER 1955 The physiology and biochemistry of the essential fatty acids. Vitamins and Hormones, *13:* 30.

DRYDEN, L. P., P. F. GLEIS AND A. M. HARTMAN 1956 Reproduction and lactation of rats fed glyceryl trilaurate-containing diets. J. Nutrition, *58:* 335.

DUMAS, J. B. A. 1871 Note sur la constitution du lait et du sang. Ann. Chim. et Phys. Anat. Ser., *22:* 445.

EL RIDI, M. S., W. M. AZOUZ AND A. ABDEL HAY 1955 Über die Wirkung von Squalen auf die Förderung der Laktation von Ratten bei Fütterung mit gereinigter squalenfeier Nahrung. Z. Physiol. Chem., *299:* 283.

ELVEHJEM, C. A. 1956 The effects of amino acid imbalance on maintenance and growth. In Cole, W. H., Editor, Some aspects of amino acid supplementation. Rutgers Univ. Press, New Brunswick, New Jersey.

GANDER, J. E., AND M. O. SCHULTZE 1955 Concerning the alleged occurrence of an "animal protein factor" required for the survival of young rats. I. Studies with unpurified rations. J. Nutrition, *55:* 543.

GREENSTEIN, J. P., S. M. BIRNBAUM AND M. WINITZ 1956 A water-soluble synthetic diet. Arch. Biochem. Biophys., *63:* 266.

GUERRANT, N. B., G. P. WHITLOCK, M. L. WOLFF AND R. A. DUTCHER 1947 Response of rats to diets containing varying amounts of glycerol and of propylene glycol. Bull. National Formulary Committee, *15:* 205.

HALLANGER, L. E., AND M. O. SCHULTZE 1956 Development of fatty livers during lactation of rats fed amino acid rations. J. Nutrition, *60:* 25.

HARPER, A. E. 1956 Amino acid imbalances, toxicities and antagonisms. Nutrition Rev., *14:* 225.

HEDIN, P. A., AND M. O. SCHULTZE 1955 Maternal diet and other factors affecting the lipid content of livers of very young rats. J. Nutrition, *56:* 129.

HOLMAN, R. T., AND S. ENER 1954 The use of urea-inclusion compound containing essential fatty acid in an experimental diet. Ibid., *53:* 461.

KEANE, K. W., E. M. COHN AND B. C. JOHNSON 1951 Reproductive failure of rats on glyceryl trilaurate-containing diets and its prevention by certain natural fats. Ibid., *45:* 275.

LEWIS, P. K., W. G. HOEKSTRA, R. H. GRUMMER AND P. H. PHILLIPS 1956 The effect of certain nutritional factors including calcium, phosphorus and zinc on parakeratosis in swine. J. Animal Sc., *15:* 741.

LOOSLI, J. K., J. F. LINGENFELTER, J. W. THOMAS AND L. A. MAYNARD 1944 The role of dietary fat and linoleic acid in the lactation of the rat. J. Nutrition, *28:* 81.

MACKENZIE, C. G., J. B. MACKENZIE AND E. V. MCCOLLUM 1939 Growth and reproduction on a low fat diet. Biochem. J., *33:* 935.

MORUZZI, G., M. MARCHETTI, R. VIVIANI AND A. RABBI 1956 Acido orotico e fattori proteici animali della caseina. Intern. Z. Vitaminf., *16:* 328.

PHILLIPS, W. A., AND C. P. BERG 1954 Effect upon growth of the D isomers in synthetic mixtures of the essential amino acids. J. Nutrition, *53:* 481.

PICCIONI, M., A. RABBI AND G. MORUZZI 1951 Animal protein factor for the rat present in crude casein and its relationships with vitamin B_{12}. Science, *113:* 179.

SCHULTZE, M. O. 1956 Reproduction of rats fed protein-free amino acid rations. J. Nutrition, *60:* 35.

SCHWARZ, K. 1954 Factors protecting against dietary necrotic liver degeneration. Ann. New York Acad. Sci., *57:* 878.

WRETLIND, A. 1956 The utilization of D-valine for growth in rats. Act. Physiol. Scand., *36:* 119.

PARTITION OF CALCIUM METABOLISM IN DAIRY COWS[1]

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The mechanisms by which an animal meets the stresses of a constantly changing environment are, in general, not well known. However, the existence of such mechanisms has long been regarded as a necessity for life. This study is concerned with the adaptive mechanisms associated with calcium balance in the dairy cow. Calcium balance, or imbalance, in the cow results from the difference between the absorption of dietary calcium and the secretion of metabolic fecal calcium.

The means for an evaluation of absorption rate as distinct from the difference between intake and fecal loss was provided by the work of Hevesey ('48) and of Kleiber et al. ('51). Calcium balance trials gave no more than the overall efficiency of calcium utilization.

Using these new techniques, Kleiber et al. ('51) with dairy cattle and Lofgreen and Kleiber ('53) with lambs noted differences in the "true digestibility" of phosphorus which could be correlated with the level of food intake. Visek et al. ('52) showed that the availability of dietary calcium is a function of physiological need. They noted a positive correlation between lactation rate and absorption of dietary calcium in goats. Similar results have been obtained with cattle (Hansard et al., '54) and with rats (Hansard and Plumlee, '54).

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These determinations are based upon a comparison of the level of radioactivity in the serum and feces. We noted in our first trials, Ca-3 and Ca-4, that a single, intravenous, injection of 10 microcuries of Ca^{45} per kilogram of body weight will yield serum and fecal samples of sufficiently high count rates for accurate radioassay more than 90 days after injection.

Thus it was possible to study the effects of changes in physiological status, e.g. pregnant vs. non-pregnant, lactating vs. non-lactating, and in the plane of nutrition, on absorption and secretion within the same cow following a single injection of isotope.

TABLE 1

General data concerning the experimental animals

COW NO.	TRIAL NO.	AGE	WEIGHT	MILLICURIES INJECTED	DURATION OF TRIAL AFTER INJECTION
		yrs.	*kg*		*days*
9	Ca-3	3.8	434	4.60	69
20	Ca-4	3.0	513	4.50	82
9	Ca-6	4.0	477	4.80	34
20	Ca-7	3.3	545	4.75	75
33	Ca-10	3.3	400	4.00	184
44	Ca-11	3.3	430	4.30	82
63	Ca-12	2.5	396	4.00	150
17	Ca-13	4.8	463	4.63	97

EXPERIMENTAL

Four sterile and two pregnant Jersey cows were used for this study. They were fed individually in stanchions during a pre-injection period of at least 60 days duration. Each cow received approximately 5 millicuries of radiocalcium (Ca^{45}) intravenously by means of a jugular cannula (Ralston et al., '49). Details pertaining to the cows are listed in table 1.

The ration each cow received during this period is listed in table 2. The diets furnished somewhat less than the recommended allowances for dairy cows as listed in the National Research Council Bulletin 3, 1945. It was noted however, throughout the trials, that the cows maintained

body weight and that they did not exhibit symptoms of malnutrition or undernutrition. During the lactation periods of cow 20 (trial Ca-7), the daily plane of nutrition was substantially increased (table 6).

During the collection periods following the injection of isotope, the cows were housed in the large animal respiration chamber (Kleiber, '35). Blood, urine and fecal samples were taken at regular intervals after the injection. Urine and feces were collected quantitatively each day. They were pooled, weighed and sampled. All the samples were analyzed for

TABLE 2

Composition of the daily diet during the steady state

TRIAL	COW	OAT HAY	BARLEY	SUPPLEMENT	Ca/P RATIO	PHYSIOLOGICAL STATE
		lb.	*lb.*	*gm*		
Ca-3	9	3	6	$CaCO_3$, 21	1/1	Pregnant
Ca-4	20	3	6	NaH_2PO_4, 55	1/7	Pregnant
Ca-6	9	3	6	$CaCO_3$, 21	1/1	Pregnant
Ca-7	20	3	6	NaH_2PO_4, 55	1/7	Pregnant
Ca-10	53	3	6	$CaCO_3$, 21	1/1	Sterile
Ca-11	44	3	6	NaH_2PO_4, 55	1/7	Sterile
Ca-12	63	3	6	$CaCO_3$, 150	6/1	Sterile
Ca-13	17	3	6	$CaCO_3$, 75	5/1	Sterile

total calcium and for radioactive calcium after the methods described by Comar et al. ('51). Metabolic fecal calcium was calculated after Kleiber et al. ('51).

RESULTS AND DISCUSSION

The first series of digestion trials to be reported was conducted with 4 sterile Jersey cows. These cows had been on a daily ration of 3 lb. of oat hay, 6 lb. of barley, and a supplement of either calcium carbonate or sodium phosphate for a minimum of three months before, and during, the trials. The results which are listed in table 3 show that on the very low Ca/P rations, the cows were in negative calcium balance

and that only when the Ca/P ratio exceeded unity was positive calcium balance achieved.

Furthermore, with increasing calcium intake (and an increasing Ca/P ratio), there was an increase in the amount of calcium absorbed from the gut. This increase ranged from zero to 37 gm/day and led to an increase in "true digestibility" from zero to 58%. There was a marked decrease in the metabolic fecal calcium with increasing calcium intake. The cow on the 1/1Ca/P ration secreted 6.0 gm calcium/day

TABLE 3

Effect of dietary calcium and phosphorus levels on calcium absorption and balance

Comparison of 4 sterile cows [1]

TRIAL	Ca-11	Ca-10	Ca-13	Ca-12
Cow no.	44	33	17	63
Mineral intake, gm/day				
Calcium	3.8	12	33	64
Phosphorus	25	11.5	7.0	11.5
Supplement	55 (NaH_2PO_4)	21 ($CaCO_3$)	75 ($CaCO_3$)	150 ($CaCO_3$)
Ratio: Ca/P	1/7	1/1	5/1	6/1
Ratio: ϵ/π [2]	0.70	0.33	0.28	0.12
Fecal calcium, gm/day				
Undigested		12.0	14.4	27.3
Metabolic		6.0	5.6	3.7
Total	17	18.0	20.0	31.0
Calcium absorbed, gm/day		0	+ 19	+ 37
Calcium balance, gm/day	— 14	— 6	+ 13	+ 33
True digestibility, %		0	58	58
Relative secretion,[3] %		50	17	5.8

[1] We were unable to calculate the metabolic fecal and absorbed calcium for trial Ca-11 because the cow went "off feed" during the collection period. Calcium balance had been measured during an earlier collection period when the cow was eating normally.

[2] ϵ/π is the ratio of excreta calcium specific activity to serum calcium specific activity where specific activity is expressed as microcuries Ca^{45} per gram atom of calcium.

[3] (Metabolic fecal Ca/Ca intake) × 100.

into the feces (50% of the intake), whereas the cow on the high Ca/P diet secreted 3.7 gm/day into the feces (5.8% of the intake).

It was noted that the increase in the "true digestibility" (58%) was nearly the same as the decrease in relative secretion rate of metabolic fecal calcium (44%) between cows fed 1/1 and 6/1 Ca/P rations. However, the change in absorption rate (37 gm/day) is much greater than the change in fecal secretion rate (2.3 gm/day).

TABLE 4

Partition of daily dietary calcium during a consecutive series of digestion trials with cow 33 (Trial Ca-10)

Period	1	2	3	4	5	6	7	8
Duration, days								
Precollection	60	0	7	0	10	0	0	0
Collection	21	14	8	7	3	7	7	6
Mineral intake								
Ca, gm	12	12	3.8	3.8	76	76	3.8	3.8
P, gm	11.5	11.5	11.5	11.5	11.5	11.5	25.5	25.5
Ratio: Ca/P	1/1	1/1	1/3	1/3	7/1	7/1	1/7	1/7
Ratio: ϵ/π		0.33	0.43	0.35	0.11	0.08	0.36	0.55
Fecal calcium, gm								
Undigested		13.0	7.8	10.7	68.0	48.0	10.6	4.2
Metabolic		6.7	6.0	5.7	8.0	4.0	5.9	5.2
Total	17.0	19.7	13.8	16.4	76.0	52.0	16.5	9.4
Calcium absorbed, gm		0	−4.0	−6.9	+8.0	+52	−7.2	0
Calcium balance, gm	−5	−7	−10	−13	0	+24	−13	−5.6
True digestibility, %		0	—	—	10	69	—	0

These responses on the part of the cow seem contrary to the usual concept of adaptation. We had anticipated that our cows, like those of Visek et al. ('53), would utilize dietary calcium more efficiently when on a low-calcium diet. It seems likely that the responses we noted were elicited by the high phosphate content of the ration.

Table 4 lists the results of a series of digestion trials which were conducted on sterile cow 10 over a period of 13 weeks. As in the earlier trials, positive calcium balance was achieved only when the dietary Ca/P ratio exceeded unity and the loss of metabolic fecal calcium varied less than absorption from the gut. These trials reveal considerable information not only on the relative importance of absorption and secretion on calcium balance, but perhaps more important, on the rate at which the cow adapts to a change in diet.

Throughout the 12-week preinjection period, and during the subsequent 5-week postinjection period, the cow was maintained on the diet of 3 lb. of oat hay and 6 lb. of barley, which was supplemented with 21 gm of calcium carbonate. Upon completion of this period, the supplement was removed from the diet and 7 days elapsed before two consecutive collections, numbers three and 4, of 8 and 7 days respectively, were made. The results from these trials indicate that not only did the cow suffer an increased net calcium loss but also that there was an apparent negative absorption of calcium from the ration. One explanation for this paradox is a delay in passage of the previously fed, relatively high calcium diet through the digestive tract.

Following these collections, the diet was supplemented with 180 gm of calcium carbonate. Ten days elapsed before collections 5 and 6 were taken. Apparently even 10 days was insufficient time for the cow to become completely adjusted to the new diet. During the collection period 5, the cow had just achieved calcium balance (76 gm intake, 76 gm outgo) and was absorbing, on the average, only 10% of the dietary calcium. However, during collection period 6, a positive calcium balance of 24 gm/day was realized and the "truly digested" calcium amounted to 69% of the intake. Simultaneously, metabolic fecal losses decreased from 8 to 4 gm/day.

Upon completion of period 6, the dietary Ca/P ratio was changed from 7/1 to 1/7 by replacing the 180 gm calcium carbonate supplement with 55 gm of sodium phosphate. Excreta collections were begun at once. Thus no time was

allowed for adaptation to the new diet or for clearing the digestive tract of the previously-fed, high-calcium diet. This practice led to the observations listed in period 7, namely, a negative calcium balance of 13 gm/day and an apparent negative absorption of 7 gm/day. The influence of the high calcium diet was noted, but to a lesser degree during period 8.

TABLE 5

Partition of daily dietary calcium during a consecutive series of digestion trials with cow 20 (Trial Ca-7)

Period	1	2	3	4	5	6	7
Duration, days							
Precollection	90	2	5	5	5	5	5
Collection	18	4	5	3	5	4	6
Mineral intake							
Ca, gm	3.8	12	26	26	24	25	26
P, gm	26	11.5	20	20	17	15	20
Ratio: Ca/P	1/7	1/1	1.3/1	1.4/1	1.4/1	1.6/1	1.3/1
Ratio: ϵ/π	0.50	0.30	0.16	0.42	0.33	0.33	0.25
Fecal calcium, gm							
Undigested	4.1	11.0	23.8	10.7	13.0	16.1	17.4
Metabolic	4.2	4.7	4.5	7.4	6.4	7.6	5.8
Total	8.3	15.7	28.3	18.1	19.4	23.7	23.2
Ca absorbed, gm	0	1	2.2	15.3	11	8.9	8.6
Ca balance, gm	−4.5	−3.7	−2.3	+7.9	+4.6	+1.3	+2.8
True digestibility, %	0	8.3	8.5	58	46	35	33

Thus it appears that a minimum of at least two weeks should be allowed for trials of this nature between the initiation of a new diet and the start of a collection period. This would be particularly important if we wanted to measure the true digestibility of calcium in a series of feedstuffs.

A similar series of digestion trials was conducted with cow 20 over a period of 10 weeks. The results are listed in table 5. During this time, there were marked changes in the physiological and nutritional status of the cow. These changes are listed in table 6.

The first collections were made while the cow was 9 months pregnant and was eating a low-Ca/high-P diet. We note that not only was the cow in negative calcium balance (—4.5 gm/day) but also that no calcium was being absorbed from the ration.

The next collection was taken during early lactation following a two-day precollection period. A calcium supplement had been added to the ration of oat hay and barley so that the resulting Ca/P ratio was brought up to 1/1. The data indicate that some calcium was now being absorbed from the diet. However, it seems probable that considerably more

TABLE 6

Composition of the daily diet and physiological status during each period of digestion trial Ca-7

PERIOD	ROUGHAGE	CONCENTRATE	SUPPLEMENT	PHYSIOLOGICAL STATUS
	lb.	*lb.*	*gm*	
1	Oat hay, 3	Barley, 6	NaH_2PO_4, 55	Pregnant
2	Oat hay, 3	Barley, 6	$CaCO_3$, 21	Lactating
3	Alfalfa, 5	Barley, 10	0	Lactating
4	Alfalfa, 5	Barley, 10	0	Lactating
5	Alfalfa, 5	Barley, 10	Versene,[1] 214	Lactating
6	Alfalfa, 5	Barley, 6.3	Versene, 214	Dry
7	Alfalfa, 5	Barley, 10	0	Dry

[1] Disodium ethylenediamine tetraacetate.

calcium was actually being absorbed than is indicated; the results of the trials with cow 10 showed that a considerably longer period of adaptation is necessary.

Following period 2, the supplement was removed from the diet and the cow was fed 5 lb. of alfalfa hay and 10 lb. of barley. The Ca/P ratio was slightly increased. Metabolic fecal calcium loss increased somewhat but there was a much greater change in absorption (from 2.2 to 15.3 gm/day). The cow "went into" positive calcium balance. We are somewhat at loss to explain this marked change in "true digestibility" of the dietary calcium. It is possible that sufficient time had now elapsed since the previously-fed, high-phosphorus diet

to allow for complete adaptation to the new diet. On the other hand, it could reflect the higher plane of nutrition afforded by the alfalfa hay and increased barley ration. The somewhat improved Ca/P ratio of the new ration, or an actual higher "availability" of the calcium from alfalfa hay over that of the calcium from calcium carbonate and oat hay.

Next, we attempted to lower the Ca/P ratio of the above ration by adding the calcium chelating agent, disodium ethylenediamine tetraacetate — hereafter referred to by its trade name, Versene, to the feed. Versene forms a soluble complex with calcium which renders calcium physiologically unavailable. The cow was fed 214 gm of Versene daily, an amount which would chelate 23 gm of calcium (90% of intake). The cow refused to eat Versene in dry form — no doubt due to the bitter taste. This taste could not be sufficiently masked with palatable sweets such as molasses. However, the cow was apparently unable to detect the Versene when it was added to her daily water supply.

The results of this trial are listed under period 5. When compared with the absorption from the identical diet (period 4) without added Versene, they show that less calcium was being absorbed. However, it is not clear why all of the dietary calcium was not tied up. It is possible that the calcium was liberated from the calcium-Versene complex by rumen microorganisms.

Still another possibility lies in the fact that Versene-chelated calcium, in solution, is completely and instantly exchangeable (Rubin et al., '53). Thus it may be that radioactive calcium ions that are secreted into the gut are exchanged for the stable calcium ions that are chelated to the Versene. The liberated stable calcium ions could then be absorbed. This would tend to increase the specific activity of the excreta. There would be little effect on the specific activity of the serum as serum calcium is in specific activity equilibrium with a much larger pool of skeletal calcium. The net result is an increase in the ratio of excreta/serum calcium specific

activity. This would lead to the calculation of an abnormally high secretion of metabolic fecal calcium, to a decrease in undigested calcium, and ultimately, to an erroneously high "true digestibility."

At the end of period 5 milking of the cow was stopped in order to study the effect which lactation had on these processes. Five days later, collection period 6 was begun. During

TABLE 7

The effect of physiological demand upon the partition of daily dietary calcium

Trial	Ca-11	Ca-7	Ca-4	Ca-10	Ca-3
Cow no.	44	20	20	33	9
Physiological status	open	Preg. 8 mo.	Preg. 5 mo.	open	Preg. 5 mo.
Mineral intake, gm/day					
Calcium	3.8	3.8	3.8	12	12
Phosphorus	25	26	25	11.5	11.5
Ratio: Ca/P	1/7	1/7	1/7	1/1	1/1
Ratio: ϵ/π	0.70	0.50	0.63	0.33	0.33
Fecal calcium, gm/day					
Undigested		4.1	2.1	12.0	8.0
Metabolic		4.2	3.5	5.8	4.0
Total	17	8.3	5.6	17.8	12.0
Calcium absorbed, gm/day		0	+ 1.7	0	+ 4.0
Calcium balance, gm/day	— 14	— 4.5	— 1.8	— 5.5	0
True digestibility, %		0	45	0	33

the pretrial and collection period, the cow was fed the same diet, including Versene, as during period 5. The results show that concurrent with the decreased demand for dietary calcium, there was a decrease in the amount which was absorbed from the gut and a slight increase in the total fecal loss. During the final collection period 7, the chelating agent was removed from the water. There was a slight decrease in metabolic fecal loss but very little change in absorbed calcium.

A comparison of this period (non-lactating) with period 4 (lactating) shows that the lactating cow maintains a greater efficiency of calcium utilization chiefly by increasing the amount of absorbed calcium. The metabolic fecal losses of both cows were roughly the same.

The data in table 7 have been arranged to reveal the effect of physiological status on the partition of dietary and fecal calcium in two groups of non-lactating cows. The groups differed between themselves only in the Ca/P ratio of the ration. Within each group, the cows are listed from left to right according to their physiological demands for calcium. The 5-months-pregnant cow is listed as having a greater requirement than the 9-months-pregnant cow. This is in keeping with the findings of Plumlee et al. ('52) that mineralization of fetal bones reaches a maximum rate between the 4th and 7th month of gestation.

In both cases the increased calcium requirements associated with pregnancy to meet the skeletal growth of the fetus are reflected in the calcium metabolism of the dam. None of the cows which had been fed a 1/7 Ca/P ration were able to maintain calcium balance but those cows which had the greatest need for calcium (5 months pregnant) had a greater efficiency of calcium utilization (intake/outgo) than did the 9-months-pregnant cow; and both pregnant cows, in turn, made more efficient use of their dietary calcium than did the sterile cows.

It seems important to note again that absorption as well as metabolic fecal secretion of calcium responded in this adaptation. That is, as the demands for calcium increased, there was a concomitant increase in the amount of absorbed calcium and a decrease in the loss via metabolic fecal secretion. A comparison of the response of each cow to its "physiological counterpart" on the second dietary regime, shows that those cows fed a Ca/P ratio of 1/1 were better able to maintain calcium balance than those fed a 1/7 ration.

SUMMARY

A series of digestion trials was conducted with Jersey cows following a single intravenous injection of radioactive calcium. The relative importance of absorption and metabolic fecal secretion of calcium were studied. These trials were made during the dry period, during lactation and pregnancy, with changes in the calcium and phosphorus content of the ration, and with a calcium chelating agent added to the diet.

The cows were unable to maintain calcium balance when the dietary Ca/P ratio was less than unity. Cows responded to changes in calcium or phosphorus in the diet by adjusting gain (absorption) and loss (metabolic fecal secretion). The changes in absorption were much greater than in secretion.

Cows on low-calcium (high-phosphorus) diets utilized dietary calcium less efficiently than cows on higher calcium diets. This effect may be due to the high-phosphorus content of these rations.

In other respects, our results are in keeping with the concept of calcium homeostasis. The lactating cow utilized dietary calcium more efficiently than the non-lactating cow; the pregnant cow more so than the non-pregnant cow.

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LITERATURE CITED

COMAR, C. L., S. L. HANSARD, M. P. PLUMLEE AND B. F. BARRENTINE 1951 Use of calcium^{45} in biological studies. Nucleonics, *8:* 19.

HANSARD, S. L., C. L. COMAR, AND M. P. PLUMLEE 1954 The effects of age upon calcium utilization and maintenance requirements in the bovine. J. Animal Sci., *13:* 25.

HANSARD, S. L., AND M. P. PLUMLEE 1954 Effects of dietary calcium and phosphorus levels upon the physiological behavior of calcium and phosphorus in the rat. J. Nutrition, *54:* 17.

HEVESEY, G. 1948 Radioactive indicators. Interscience Publishers, Inc., New York, 556 pp.

KLEIBER, M. 1935 The California apparatus for respiration trials with large animals. Hilgardia, *9:* 1–70.

KLEIBER, M., A. H. SMITH, N. P. RALSTON AND A. L. BLACK 1951 Radiophosphorus (P^{32}) as tracer for measuring endogenous phosphorus in cow's feces. J. Nutrition, *45:* 253–263.

LOFGREEN, G. P., AND M. KLEIBER 1953 The availability of the phosphorus in alfalfa hay. J. Animal Sci., *12:* 366–371.

PLUMLEE, M. P., S. L. HANSARD, C. L. COMAR AND W. M. BEESON 1952 Placental transfer and deposition of labeled calcium in the developing bovine fetus. Am. J. Physiol., *171:* 678–686.

RALSTON, N. P., M. KLEIBER AND A. H. SMITH 1949 Venous catheterization of dairy cows. J. Dairy Sci., *32:* 889–893.

RUBIN, M., R. D. THOMAS, T. A. LITOVITZ AND C. F. GESCHICKTER 1953 Trans. Macy Conference on Metabolic Interrelations, *5:* 64.

VISEK, W. J., L. L. BARNES AND J. K. LOOSLI 1952 Calcium metabolism in lactating goats as studied with Ca-45 following oral and intravenous administration. J. Dairy Sci., *35:* 783–792.

VISEK, W. J., R. A. MONROE, E. W. SWANSON AND C. L. COMAR 1953 Determination of endogenous fecal calcium in cattle by a simple isotope dilution method. J. Nutrition, *50:* 23–33.

THE UTILIZATION OF RAW SOYBEAN MEAL PROTEIN FOR EGG PRODUCTION IN THE CHICKEN[1]

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The utilization of raw soybean meal has been studied primarily in growing animals while little attention has been given to its utilization for maintenance and productive purposes in the adult animal (see chapter by Hayward in Markley, '51).

It is well recognized that raw soybean meal, unlike the properly heated meal, exhibits inhibitory properties on the growth of chicks and rats. This growth inhibition has been related to several factors: (1) unavailability of amino acids, particularly cystine-methionine (Mitchell and Smuts, '32; Shrewsbury and Bratzler, '33; Almquist et al., '42); (2) a trypsin inhibitor (Ham and Sandstedt, '44; Almquist and Merritt, '52); (3) a toxic protein called Soyin (Liener, '53); (4) the presence of an inadequate amount of vitamin B_{12} (Frölich, '54; Baliga et al., '54). While the picture is thus a rather complex one with regard to the utilization of raw soybean meal for growth purposes, it has been observed that laying chickens will not be adversely affected when *some* raw soybean meal is incorporated into *practical* laying rations (Tomhave and Mumford, '36; Carver et al., '46). Since a differential response to raw soybean meal by the laying hen

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compared with the growing chick could yield important information on the nature and properties of the raw meal, the present studies were undertaken.

EXPERIMENTAL

Single-Comb White Leghorn hens were maintained in individual wire cages in a temperature-regulated room. Water was supplied ad libitum but feed intake was controlled in several experiments as indicated below. The experimental period was three to 4 weeks since Fisher and Johnson ('56)

TABLE 1

Basal rations

INGREDIENTS	FOR HENS	FOR CHICKS
	%	%
Glucose (cerelose)	to equal 100	to equal 100
Corn oil	3.0	3.0
Choline chloride	0.1	0.2
NaCl	0.5	0.5
Vitamins A, D and E [1]	0.1	0.1
Dicalcium phosphate	4.0	2.2
Trace mineral mix [2]	3.0	1.0
Vitamins [3]	0.15	0.15
Variable ingredients	DL-methionine Vitamin B_{12} Soybean meal (level and source) Crude soybean inhibitor	DL-methionine Soybean meal (level and source)

[1] Supplied per kilogram of diet: 10,000 IU vitamin A, 600 IU vitamin D_3 and 5 IU alpha toeopheryl acetate.

[2] Mico concentrate, Limestone Corp. of America, Newton, N. J. Contains: Mn, 1.0; iodine, 0.225; Fe, 0.175; Cu, 0.125; Zn, 0.009; Co, 0.010; Ca, 32.0%.

[3] In milligrams per kilogram of diet: Thiamine HCl, 25; riboflavin, 16; Ca pantothenate, 20; pyridoxine HCl, 6; biotin, 0.6; folic acid, 4; inositol, 100; *p*-amino benzoic acid, 2; 2-methyl 1,4-naphthoquinone, 5; ascorbic acid, 250; niacin, 150.

had shown that a two-week experimental period allowed sufficient time to demonstrate a protein or amino acid deficiency in hens. Chicks were raised in groups of 8 per lot in electrically heated batteries. They were supplied ad libitum with feed and water. In both the hen and chick experiments, semi-purified rations were used (table 1) in which the soybean meal provided the only source of dietary protein.

Raw vs. heated soybean meal

Since raw soybean meal had not been fed previously as the only source of protein for hens, the first three experiments were designed to test its utilization in otherwise adequately balanced rations. In each of these three trials, 8 hens, respectively, were fed ad libitum the basal diet supplemented with 30% (15% protein) of either a raw or a heated soybean meal.[2] It should be emphasized that in these trials liberal amounts of methionine and vitamin B_{12} were present in the basal diet. Statistical analysis of the results in table 2 indicates clearly that the protein from the raw soybean meal and the properly heated meal were equally well utilized, nor were there any differences in feed consumption which might indicate a less efficient utilization of raw soybean protein for egg production.

To ascertain if the raw soybean meal used in the hen studies contained growth inhibiting properties, chick tests were conducted simultaneously with the hen studies with the results shown in table 3. These data confirm the growth inhibition both qualitatively and quantitatively, as described by Almquist et al. ('42) on diets containing an adequate amount of methionine.

[2] Both meals were obtained through the courtesy of A. E. Staley Manufacturing Co., Decatur, Ill. The raw meal was solvent-extracted with only enough heat applied to remove excess solvent. Urease activity was in excess of 40 ml of 0.1 N HCl/gm during a 30-minute incubation period (Staley's Determination). The heated meal was prepared under standard commercial conditions.

The effect of protein level and vitamin B_{12} on raw soybean meal utilization

Since previous experiments in this laboratory had indicated that 15% of soybean meal protein was in excess of the minimum requirement for good egg production, the following factorial design was next set up in studying raw soybean meal utilization (exp. 4). As indicated in table 4, three levels of protein, 12.21 (N × 6.25), 15, and 30% were fed both with raw and properly heated soybean meal. In addition, all

TABLE 2

A comparison of raw vs. heated soybean meal for hens in diets adequate in vitamin B_{12} (0.02 mg/kg diet) and methionine (0.3% added to basal)

EXP. NO.	AVERAGE 28-DAY EGG PRODUCTION PER BIRD [1]		AVERAGE DAILY FEED CONSUMPTION DURING 28-DAY EXPERIMENTAL PERIOD	
	Raw	Heated	Raw	Heated
			gm	*gm*
1	21.8 ± 0.5 [2]	23.2 ± 0.5	115 ± 5.1	125 ± 5.6
2	17.9 ± 0.9	17.2 ± 1.6	111 ± 4.5	114 ± 4.8
3	18.0 ± 1.0	18.9 ± 1.1	127 [3]	122 [3]

[1] Eight birds in each group.

[2] Standard error of the mean.

[3] Total 28-day feed consumption only was recorded; therefore no standard error can be calculated.

TABLE 3

Demonstration of growth depression in chicks by raw vs. heated soybean meal in diets otherwise complete in vitamin B_{12} (0.02 mg/kg) and methionine (0.3% supplemental)

PROTEIN LEVEL	SOURCE OF SOYBEAN	THREE WEEKS			
		Average gain	Difference	Average feed consumed	Difference
%		*gm*	%	*gm*	%
20	Heated	314 [1]		476	
20	Raw	266	— 15.2	530	+ 11.3
23	Heated	320		475	
23	Raw	278	— 13.1	496	+ 4.4
26	Heated	321		472	
26	Raw	289	— 10.0	492	+ 4.2

[1] Average gain of duplicate 8-bird lots.

treatments were fed with and without vitamin B_{12} to the *same* animals, in two separate 4-week experimental periods. The vitamin B_{12}-free preceded the B_{12}-supplemented period and all birds were on a practical B_{12}-containing laying ration before and for two weeks between experiments.

Birds were selected in a narrow weight range and were fed 100 gm per bird per day, thus insuring an adequate

TABLE 4

The effect of protein level and vitamin B_{12} on raw soybean meal utilization for hens in a diet otherwise adequate with respect to methionine (0.3% supplemental)

PROTEIN LEVEL	SOURCE OF PROTEIN	VITAMIN B_{12}	AV. WEEKLY EGG PRODUCTION/BIRD [1]				TOTAL/28 DAYS
			1st wk.	2nd wk.	3rd wk.	4th wk.	
%							
12.21	Raw	—	3.6 (11) [2]	1.0 (0)	3.6 (2)	3.4 (4)	11.6 ± 1.8 [3]
12.21	Heated	—	5.2 (0)	3.8 (0)	4.8 (0)	4.8 (0)	18.6 ± 1.0
12.21	Raw	+	4.8 (4)	2.4 (5)	3.0 (6)	3.0 (4)	13.2 ± 1.1
12.21	Heated	+	5.0 (4)	3.6 (1)	2.8 (0)	4.4 (0)	15.8 ± 2.0
15.0	Raw	—	4.8 (3)	2.8 (0)	4.2 (0)	3.4 (0)	15.2 ± 2.5
15.0	Heated	—	4.8 (0)	3.6 (3)	4.8 (0)	3.6 (1)	16.8 ± 2.9
15.0	Raw	+	5.8 (1)	4.6 (1)	4.8 (2)	4.6 (0)	19.8 ± 1.6
15.0	Heated	+	5.4 (0)	4.6 (3)	4.2 (2)	3.6 (2)	17.8 ± 3.8
30.0	Raw	—	5.2 (0)	4.2 (2)	1.4 (0)	3.6 (0)	14.4 ± 2.1
30.0	Heated	—	5.8 (0)	5.2 (0)	4.0 (2)	3.6 (1)	17.6 ± 2.4
30.0	Raw	+	5.4 (0)	4.4 (0)	3.6 (0)	3.0 (0)	16.4 ± 2.1
30.0	Heated	+	5.0 (0)	4.4 (0)	4.0 (1)	4.4 (0)	17.8 ± 3.4

[1] Five birds per treatment group.

[2] Number of hen days on which sufficient feed had accumulated due to refusals, obviating the need for the daily 100 gm feed allotment.

[3] Standard error of the mean.

amount of nutrient intake as well as an equalized protein intake within each treatment group. This amount of feed was readily consumed except as indicated in table 4.

Variance analysis of the data in table 4 indicates a significant difference ($P < 0.05$) between the raw and the heated soybean meal in the *absence* of vitamin B_{12}. This difference manifested itself in a poorer utilization of the raw meal at all protein levels but particularly at the 12.21% level of protein. In the presence of vitamin B_{12} (and adequate methi-

onine), the raw meal was again as well utilized as the heated meal except for a slight depression at the lowest level, which indicates that amino acids other than methionine are not as readily available as in the heated meal. This same tendency was also evident in the chick trial (table 3) where a small improvement was noticeable with increased levels of raw soybean meal protein.

TABLE 5

The effect of methionine and soybean inhibitors on soybean protein utilization by hens fed rations containing 15% soybean protein and adequate in vitamin B_{12}

SUPPLEMENTAL DL-methionine	SOURCE OF SOYBEANS	AV. WEEKLY EGG PRODUCTION/BIRD [1]			TOTAL/21 DAYS
		1st wk.	2nd wk.	3rd wk.	
%					
0	Raw	3.2 (6) [2]	0.8 (2)	2.5 (3)	6.5 ± 1.6 [3]
0	Heated	4.2 (5)	3.0 (5)	1.5 (2)	8.8 ± 1.6
0	Heated [4]	5.0 (0)	5.0 (0)	1.8 (1)	11.8 ± 0.8
0.1	Raw	4.5 (4)	2.8 (0)	4.2 (1)	11.5 ± 2.7
0.1	Heated	4.8 (0)	4.8 (1)	3.5 (1)	13.0 ± 0.9
0.2	Raw	5.0 (4)	2.7 (1)	3.3 (3)	11.0 ± 4.1
0.2	Heated	5.0 (0)	4.5 (0)	2.8 (0)	12.2 ± 2.6

[1] Four birds per treatment group.

[2] Number of hen days on which sufficient feed had accumulated due to refusals, obviating the need for the daily 100 gm feed allotment.

[3] Standard error of the mean.

[4] This group received 0.05% crude soybean inhibitors.

The effect of methionine and crude soybean inhibitors on raw soybean meal utilization

In experiment 5, three levels of supplemental methionine were fed both with the raw and heated soybean meals. A 7th group was added which received the heated meal without supplemental methionine and in addition 0.05% of crude soybean inhibitors.[3] The protein level in all rations was maintained at 15% since this had been shown to be adequate when properly supplemented. Four hens were used per treatment and the duration of the experiment was three

[3] Crude Soybean Inhibitor S5302, Worthington Biochemicals, Freehold, N. J.

weeks. As in the vitamin B_{12} experiments, feed intake was regulated (by daily allotment) at 100 gm per bird per day, which was fully consumed except as indicated in table 5.

The results in table 5 suggest that the methionine and cystine in the raw meal are not readily available. Good production was not maintained even with 0.2% methionine supplementation in contrast to the heated meal where production was significantly better ($P = 0.05$) with only 0.1% added methionine. Since the highest level of supplemental methionine was 0.2%, it can only be inferred on the basis of the previous experiments that good production would have resulted from 0.3% supplementation. The addition of soybean inhibitors in no way interfered with egg production. In fact, the birds on that diet laid more eggs than those without it, even though they did slow down during the third week due to the methionine deficiency, as did the comparable group without inhibitors.

DISCUSSION

It appears from the studies herein reported that raw soybean meal protein can serve as the only source of dietary protein for egg production provided it is fed at a level exceeding the minimum protein requirement for the heated meal and the diet is otherwise properly supplemented with sulfur amino acids and vitamin B_{12}. Since the growing chick is still inhibited under the above conditions, it would appear that the laying hen is insensitive to the toxic and trypsin-binding principles in raw meal. Except for this insensitivity, the raw soybean protein is just as inferior in terms of the availability of sulfur and other amino acid(s) for the hen as it is for the growing chick. This was exemplified by the need for more protein, methionine and vitamin B_{12} when the raw soybean meal was compared to properly heated meal.

In another experiment, the details of which are omitted, it was shown that developing pullets through 14 weeks of age still cannot utilize raw soybean protein as well as the

heated meal (when fed diets properly supplemented with methionine and vitamin B_{12}). Apparently, then, the insensitivity to the growth inhibitors of the raw meal does not assert itself until a later age.

As was previously pointed out (Fisher and Johnson, '56), birds receiving a suboptimal level of protein or amino acid immediately respond with a lowered feed intake. This observation was made again in experiment 4 (table 4), and experiment 5 (table 5). Because of space limitations the detailed daily egg production and feed consumption for these groups are not given but the interesting observation was made that after a few days of partial feed refusal (see tables 4 and 5) egg production in many birds stopped completely for approximately one week. During this week these birds, even though they were not laying any eggs, readily consumed all the feed offered. After that week, egg production again commenced and again the birds began to refuse part of the daily feed allotment.

This observation suggests that on suboptimal amino acid intake, a bird in egg production refuses to eat until the stress of production has ceased. As her requirements for amino acids approach those necessary for maintenance only, the diet becomes adequate and the hen begins to eat and store protein toward the time that egg production will again commence. As production resumes, the lack of an adequate daily intake of amino acids again forces the hen out of production. Observations of this cycle are best apparent in the early stages of an experiment since most hens are similarly affected at that time; during prolonged experiments, individual adaptations produce cycles of varying lengths among a hen population.

The above observations would indicate that the mechanism of vitamin B_{12} action was to enhance the utilization of amino acids from the raw soybean meal. The same cycle of alternate feed refusal and egg production was observed in the absence of vitamin B_{12} as was the case when an inadequate amount of methionine was fed.

The study on the effect of methionine on the utilization of raw soybean meal permits a calculation of the methionine requirement of the hen. The 15% protein diet with the properly heated meal contains by calculation 0.20% of methionine and 0.21% of cystine. Excellent egg production was maintained with the 0.1% DL-methionine supplementation, giving a total of 0.3% of methionine. This value agrees closely with the value of 0.28% reported by Leong and McGinnis ('52) in the presence of a similar amount of cystine (0.25%) in the diet.

Finally, some speculation is in order concerning the insensitivity of the hen to the trypsin and other inhibitors. Perhaps the secretion of trypsin is considerably greater in the hen than in the growing chick thus providing an excess beyond that which is bound up with the inhibitor. It is also conceivable that the time factor for the simultaneous presence of all essential amino acids (Geiger, '47) is not so critical for egg production as it is for growth thereby permitting greater utilization of those amino acids which are absorbed in the large intestine. Carroll et al. ('52) showed that unlike the nitrogen from properly heated meal which is entirely absorbed from the small intestine, substantial amounts of nitrogen from raw meal are absorbed from the large intestine.

SUMMARY AND CONCLUSIONS

The utilization of raw soybean meal as the only source of dietary protein was studied in laying hens in experiments of three- and 4-weeks duration. It was shown that raw meal when fed at the 15% protein level and properly supplemented with methionine and vitamin B_{12} would support equally good egg production as properly heated meal under the same conditions.

When the effects of protein level, vitamin B_{12}, and methionine and crude soybean inhibitor were studied in separate trials it was shown that: (1) the raw meal was poorly utilized at the 12.21% protein level; this level supported good pro-

duction with the heated meal; (2) supplementary vitamin B_{12} was essential for proper utilization of raw soybean meal irrespective of protein level but was not essential with properly heated meal; (3) the utilization of sulfur amino acids from the raw meal was inferior to that from heated meal; (4) hens were insensitive to the growth-inhibiting properties of crude soybean inhibitors. It is concluded that the major difference in the utilization of raw soybean meal between laying hens and growing chicks lies in the insensitivity of the former to soybean inhibitors. Thus, when raw meal was fed at a sufficiently high protein level and properly supplemented with methionine and vitamin B_{12}, it was effectively utilized as a good protein source for egg production purposes under the conditions of these experiments.

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LITERATURE CITED

ALMQUIST, H. J., E. MECCHI, F. H. KRATZER AND C. R. GRAU 1942 Soybean protein as a source of amino acids for chicks. J. Nutrition, *24:* 385.

ALMQUIST, H. J., AND J. B. MERRITT 1952 Effect of soybean antitrypsin on growth of the chick. Arch. Biochem., *35:* 352.

BALIGA, B. R., S. BALAKRISHNAN AND R. RAJAGOPALAN 1954 Biological value of proteins as influenced by dietary vitamin B_{12}. Nature, *174:* 35.

CARROLL, R. W., G. W. HENSLEY AND W. R. GRAHAM, JR. 1952 The site of nitrogen absorption in rats fed raw and heat-treated soybean meals. Science, *115:* 36.

CARVER, J. S., J. MCGINNIS, C. F. MCCLARY AND R. J. EVANS 1946 The utilization of raw and heat-treated soybean oil meal for egg production and hatchability. Poultry Sci., *25:* 399.

FISHER, H., AND D. JOHNSON, JR. 1956 The amino acid requirement of the laying hen. I. The development of a free amino acid diet for maintenance of egg production. J. Nutrition, *60:* 261.

FRÖLICH, A. 1954 Relation between the quality of soybean oil meal and the requirements of vitamin B_{12} for chicks. Nature, *173:* 132.

GEIGER, E. 1947 Experiments with delayed supplementation of incomplete amino acid mixtures. J. Nutrition, *34:* 97.

HAM, W. E., AND R. M. SANDSTEDT 1944 A proteolytic inhibiting substance in the extract from unheated soy bean meal. J. Biol. Chem., *154:* 505.

LEONG, K. C., AND J. MCGINNIS 1952 An estimate of the methionine requirement for egg production. Poultry Sci., *31:* 692.

LIENER, I. E. 1953 Soyin, a toxic protein from the soybean. I. Inhibition of rat growth. J. Nutrition, *49:* 527.

MARKLEY, K. S. 1951 Soybeans and soybean products. Interscience Publishers, Inc., New York, Vol. 2, p. 891.

MITCHELL, H. H., AND D. B. SMUTS 1932 The amino acid deficiencies of beef, wheat, corn, oats and soy beans for growth in the white rat. J. Biol. Chem., *95:* 263.

SHREWSBURY, C. L., AND J. W. BRATZLER 1933 Cystine deficiency of soybean protein at various levels, in a purified ration and as a supplement to corn. J. Agr. Res., *47:* 889.

TOMHAVE, A. E., AND C. W. MUMFORD 1936 Ground soybeans as a protein supplement for laying birds. University of Delaware, Agr. Exp. Station Bull. No. 197.

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