Calcium Utilization and Feed Efficiency in the Growing Rat as Affected by Dietary Calcium, Buffering Capacity, Lactose and EDTA [1]

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ABSTRACT An experiment was designed to study the relationships of calcium, buffering capacity (BC), lactose and ethylendiaminetetraacetic acid (EDTA) in the nutrition of the growing rat. For the replicated, factorially arranged experiment, which involved calcium (0.19, 0.48 and 0.78%), buffering capacity (BC 1, 0.46, BC 2, 0.54 and BC 3, 0.76 ml of 1 N HCl required to reduce the pH of 5.0 g of the diet by one pH unit), lactose (0 and 12%), and EDTA (0 and 0.2%), 152 rats were used. Dihydrogen-to-monohydrogen phosphate ratios were varied to produce the different BC's, and a constant phosphorus (0.4%) was provided for all diets. As dietary calcium increased from 0.19 to 0.48%, retention and deposition of calcium in the total body and in the femur and efficiency of feed conversion showed improvement. However, 0.78% calcium did not prove beneficial over 0.48% calcium. The diets which produced the most acidic condition (BC 1) in the stomach, resulted in the most improved calcium retention, feed intake and efficiency of feed conversion. As judged from the reduced fecal calcium with BC 1 diets, the calculated increase in urinary calcium was not great enough to offset the increased calcium absorption in the intestine. Lactose increased calcium retention and improved bone calcification, and, for the total 6-week experimental period, EDTA decreased the retention and deposition of calcium.

The percentage of calcium retention based on body analysis (1) or fecal analysis (2, 3) has been found to decrease when calcium intake increased. Total calcium retained by rats with low stores of this element was considerably greater than that retained by rats with large mineral reserves, and before intake of calcium showed a greater influence on the amount of calcium retained than did current intake (4). Since feeding high levels of calcium was found to increase (5) net retention with a marked reduction in efficiency, calcium requirement values might be more of a reflection of dietary pattern than of absolute requirements.

Total body calcium in the growing rat increased (1, 6) when dietary calcium increased from 0.1 to 0.64%. Both tibia calcium and ash increased as calcium intake was increased from 0.13 to 0.76% (7). When dietary calcium for the rat (8) was increased from 0.2 to 0.35%, more efficient utilization of food was noticed. When, however, dietary calcium for growing pigs was increased from 0.48 to 0.88 to 1.32%, average daily gain and feed efficiency were decreased significantly but there was no significant effect on feed intake (9).

While in dogs (10) ingestion of an acidic diet increased urinary calcium excretion, neither calcium balance nor calcium retention was affected. In man, calcium retention was somewhat increased with basic diets but not with neutral or acidic diets. As a result of feeding an acidic diet (11), the reduced fecal calcium was not sufficient to offset the increased urinary calcium. When acidic, neutral, or alkaline diets were fed to rachitic rats, the neutral diets produced the most severe tetany (12). Gastric pH was of secondary importance in stomach emptying (13); however, decreased dietary pH increased the rate of acidification of digesta in the stomach and indirectly the rate of digestion and disappearance of nutrients from the stomach (14).

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Calcium in the milk was more efficiently retained than from other organic sources (15), and this was attributed to the lactose content of milk (16). The degree of maturity of the animal played a role in determining the favorable effect of lactose. Feeding 25% lactose accelerated bone calcification and calcium retention in rats (17), and in children (18), when compared with cod liver oil, increased calcium balance. Lactose at 12% with 0.6% calcium was considered the best diet for improved calcium retention in rats (19). Total body calcium and ash in rats (20, 21) and tibia ash in chicks (22) increased with added dietary lactose. Lactose improved efficiency of feed conversion only with ad libitum feeding (17, 20).

Urinary calcium output following an intraperitoneal injection of Ca-EDTA was about 70% of the injected dose. Orally administered Ca-EDTA was not available to the rat,[3] but when 0.3 or 1.5% Na-EDTA was fed for 112 days with a calcium-adequate diet, or 0.01% Na-EDTA with a calcium-deficient diet (23), there was no effect on calcium excretion.

The present study reports the effects of dietary calcium, buffering capacity (BC), lactose, and EDTA on calcium retention based on total carcass analysis; on fecal and urinary calcium excretion; on calcium deposition in the body and femur; and on weight gain, feed intake, and efficiency of feed conversion.

PROCEDURE

A randomized block design using 4 replicates with a $3 \times 3 \times 2 \times 2$ factorial arrangement of treatments was used so that all possible combinations of 3 levels of calcium, 3 BC's, and 2 levels each of lactose and EDTA were fed to growing rats. Levels were 0.19, 0.48 and 0.78% calcium, 0 and 12% lactose, 0 and 0.2% EDTA, with 0.4% phosphorus for each of the 36 diets. BC's were 0.46, 0.54 and 0.76 and were obtained by varying the dihydrogen to monohydrogen phoshate ratios from 1:0 (BC 1) to 1:1 (BC 2) to 1:2 (BC 3), respectively. Dietary BC was defined as milliliters of 1 N HCl required to reduce the pH of 5.0 g of the diet suspended in 45 ml of distilled deionized water by one pH unit.

TABLE 1

Composition of basal diet

	%
Sucrose	30.0
Starch	30.0
Casein	20.0
DL-Methionine	0.2
Corn oil [1]	5.0
Vitamin mixture [2,3]	2.0
Micromineral mixture [3,4]	0.03
Macromineral mixture (BC 1, 2 or 3) [5]	12.77

[1] Santoquin (Monsanto Company, St. Louis) added to corn oil to make 0.01% in diet.
[2] The vitamins mixed with dextrose supplied the following per 100 g of diet: vitamin A, 1800 IU; and vitamin D, 200 IU; and (in milligrams) α-tocopherol, 10.0; ascorbic acid. 90.0; inositol, 10.0; choline chloride, 150.0; riboflavin, 2.0; menadione, 4.5; *p*-aminobenzoic acid, 10.0; niacin, 9.0; pyridoxine·HCl, 2.0; thiamine, 2.0; Ca pantothenate, 6.0; biotin, 0.04; folic acid, 0.18; and vitamin B_{12}, 0.003.
[3] NRC (36) requirements were met.
[4] Micromineral mixture supplied the following per 100 g of diet: (in milligrams) iron, as Fe_2O_3, 8.58; manganese, as $MnCO_3$, 4.0; zinc, as $ZnCO_3$, 5.0; copper, as $CuCO_3$, 1.17; cobalt, as $CoCO_3$, 0.04; molybdenum, as $Na_2MoO_4 \cdot 2H_2O$, 0.06; iodine, as KI, 0.02; selenium as H_2SeO_5, 0.01 and fluoride, as NaF, 0.001.
[5] Refer to table 2.

Hooded Norway male rats ranging in weight from 70 to 90 g were adjusted to a mixture of laboratory animal ration and the basal diet for 1 week before feeding the appropriate experimental diets, compositions of which are shown (tables 1 and 2). Experimental diets and distilled water were supplied ad libitum for the 6-week experimental period. Rats were housed individually in stainless steel cages in a room maintained at 25° ± 1° and 57 ± 3% humidity. For each rat the dietary intake was recorded, and feces were sampled daily and frozen until analyzed for calcium and chromic oxide. The chromic oxide was added to the diets to serve as a reference substance for the determination of total fecal calcium (24, 25).

At the termination of the experiment, rats were killed by heart puncture. The digestive tract, the 2 kidneys, and the heart were removed. The empty carcass of each rat was dried to a constant weight in a forced draft oven at 65°, ether extracted and redried. The right femur was picked from the body for separate ash and calcium analyses. The remainder of the empty carcass was ground to pass a 1-mm screen in a Wiley mill and mixed; representative samples were analyzed for ash and calcium.

[3] Alexander, R. L. 1959 The availability of orally administered calcium. Ph.D. Thesis. Georgetown University, Washington, D. C.

TABLE 2

Composition of the macromineral mixtures

	Buffering capacity [1]		
	BC 1	BC 2	BC 3
	mg/100 g of diet		
Calcium acetate [2]	—	400	500
Sodium phosphate, monobasic	569	—	—
Potassium phosphate, monobasic	855	854	676
Sodium phosphate, dibasic	—	927	920
Potassium phosphate, dibasic	—	—	240
Sodium chloride	255	—	7
Sodium acetate	200	—	—
Potassium chloride	—	110	—
Potassium acetate	143	—	—
Magnesium chloride	—	290	430
Magnesium acetate	292	118	—
Chromic oxide	250	250	250
Nonnutritive bulk [3]	10,206	9,821	9,747
Total [4]	12,770	12,770	12,770

[1] Milliliters of 1 N HCl required to reduce pH of 5 g of mixed diet by 1 pH unit were 0.46, 0.54 and 0.76 for BC 1, BC 2 and BC 3, respectively.

[2] Calcium acetate was added to balance the acetate content of the 3 mixtures. The calcium content of the basal mixtures was considered when the calcium lactate supplement was added. Calcium lactate was added per 100 g of diet in g (BC 1) 1.09, 2.72 4.36; (BC 2) 0.59, 2.22, 3.85; and (BC 3) 0.47, 2.11, 3.73 to give 0.19, 0.48 and 0.78% calcium for each macromineral mixture. Calories from lactate were balanced with sucrose by replacing nonnutritive bulk.

[3] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[4] The 3 mineral mixtures supplied the following per 100 g of diet: (in milligrams) sodium, 300; potassium, 300; magnesium, 51; phosphorus, 400; chloride, 15≤; and acetate, 340.

The fecal samples from each rat were dried for 12 hours, ground, mixed and digested with nitric acid (26). Then chromic oxide (27) and calcium [4] content were determined. Dried, fat-free tissues were ashed at 600° for 8 hours and the femurs for 12 hours. The ash of the femur and of a 1-g carcass sample were each dissolved in diluted HCl, and the calcium content was determined (28, 29).

From the live weight and the total carcass calcium values of 2 rats selected at random and killed at the start of each replicate, a prediction equation was obtained which related total calcium of empty carcass to live body weight. An estimate of initial empty carcass calcium (Y) in grams of each experimental animal was obtained from the initial body weight (X) in grams as follows: $Y = 0.00808X - 0.21032$ ($S_{y.x} = \pm 0.014$). The calcium retained for the experimental period was obtained, and by relating this value to the total calcium intake a coefficient of retention was calculated. Calcium losses via the urine were calculated by subtracting fecal from dietary calcium in order to account for the total calcium intake during the experimental period. The data were subjected to analysis of variance and orthogonal comparisons for a factorial experiment (30).

RESULTS AND DISCUSSION

Although the absolute amount of retained calcium increased with increased calcium intake (table 3), the calcium retention, when expressed as a percentage of dietary calcium, decreased ($P < 0.01$) in linear and quadratic terms. The increased (linear, $P < 0.01$) fecal calcium and urinary calcium as calculated with increased dietary calcium could account for the observed reduction in the percentage of calcium retention (fig. 1). The reduction in fecal calcium per unit body weight was considered as the major factor in the increased percentage of calcium retention with low calcium intake (4, 31). The reduced fecal calcium indicated an increase in efficiency of calcium absorption in the gastrointestinal tract.[5] Decreased dietary calcium widened the urinary-to-fecal calcium ratio. Values were 1:6.7, 1:2, and

[4] Steckel, J. E., and R. L. Flannery 1965 Automatic determination of phosphorus, potassium, calcium and magnesium in wet digestion solutions of plant tissue. Technicon Symposium, Technicon Instrument Company Publications, Chauncey, New York.

[5] Ali, R. A. M., and J. L. Evans 1966 Calcium absorption from gastrointestinal segments. J. Animal Sci., 25: 888 (abstract).

TABLE 3
Calcium utilization

Criterion	Calcium, %			Buffering capacity [1]			EDTA, %		Lactose, %		SE
	0.19	0.48	0.78	0.46	0.54	0.76	0	0.2	0	0.12	
Calcium intake, g/6 weeks	1.18	2.84	4.13	2.85	2.70	2.60	2.78	2.66	2.72	2.71	0.11
Retained calcium,[2] g/6 weeks	0.82	1.28	1.24	1.27	1.10	0.99	1.22	1.01	1.07	1.16	0.09
Fecal calcium, g/6 weeks	0.34	1.03	1.72	0.94	1.04	1.13	1.00	1.06	1.08	0.99	0.05
Urinary calcium,[3] g/6 weeks	0.05	0.52	1.15	0.64	0.56	0.48	0.56	0.59	0.57	0.56	0.04

[1] Values represent BC 1, BC 2, and BC 3, respectively, as milliliters of 1 N HCl required to reduce pH of 5 g of the mixed diet by one pH unit.
[2] Significant interactions on calcium retention as grams per 6 weeks (A) and percentage of intake (B).

1. CA × BC, linear (L) × L ($P < 0.05$) and quadratic by L ($P < 0.01$)

A. Ca, %	BC 1	BC 2	BC 3
0.19	0.90	0.84	0.74
0.48	1.43	1.28	1.15
0.78	1.48	1.19	1.08

B. Ca, %	BC 1	BC 2	BC 3
0.19	74	73	63
0.48	48	45	42
0.78	34	29	28

2. Ca × Lactose, L × L ($P < 0.05$)

A.	Lactose	
Ca, %	0%	12%
0.19	0.76	0.89
0.48	1.18	1.39
0.78	1.27	1.22

B.	Lactose	
Ca, %	0%	12%
0.19	65	74
0.48	42	48
0.78	31	29

3. BC × Lactose, L × L ($P < 0.01$)

A.	Lactose	
	0%	12%
BC 1	1.13	1.41
BC 2	1.10	1.10
BC 3	0.99	0.98

B.	Lactose	
	0%	12%
BC 1	47	57
BC 2	47	51
BC 3	44	44

4. BC × EDTA, L × L ($P < 0.05$)

A.	EDTA	
	0%	0.2%
BC 1	1.42	1.12
BC 2	1.18	1.02
BC 3	1.08	0.90

B.	EDTA	
	0%	0.2%
BC 1	58	46
BC 2	52	46
BC 3	47	42

[3] Urine data were calculated by difference.

1:1.5, respectively, for calcium levels of 0.19, 0.48 and 0.78%, which assured minimal losses of calcium when dietary supply was limited.

Total body ash and calcium increased ($P < 0.01$) by increasing dietary calcium from 0.19 to 0.48%. No corresponding increase was noticed when dietary calcium was increased from 0.48 to 0.78% (table 4), due to decreased weight gain with 0.78% calcium in the diet. There appeared to be no trend toward beneficial effects regarding total and percentage femur ash and calcium (table 4) to the animal by increasing dietary calcium to 0.78%. Increasing dietary calcium from 0.19 to 0.48% increased weight gain with almost no change in feed intake (table 4), with the consequent result of increased feed efficiency. But, when dietary calcium was increased to 0.78%, there was a greater decrease in weight gain (quadratic, $P < 0.05$) than in feed intake (table 4), and this resulted in a lower feed efficiency. The decreased feed intake with increased dietary calcium controlled the amount of calcium consumed.

The increased dietary acidity represented by BC 1 increased percentage and total calcium retention ($P < 0.01$) by decreasing total facal calcium ($P < 0.01$) (fig. 1 and table 3). Urinary calcium in-

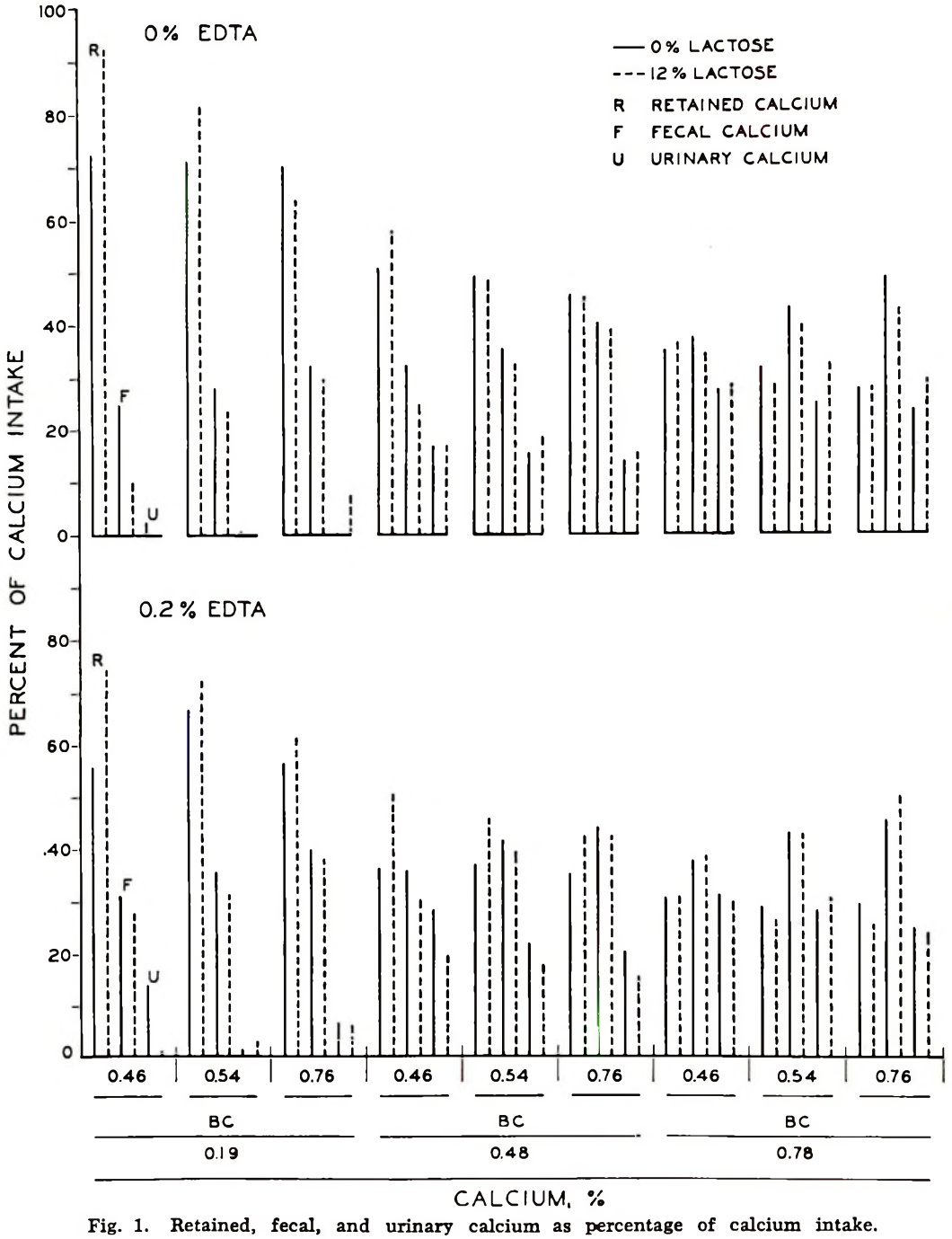

Fig. 1. Retained, fecal, and urinary calcium as percentage of calcium intake.

creased with the more acid diets ($P < 0.01$), but the increase was not large enough to overcome the decreased fecal calcium. An acidic diet was reported to decrease calcium retention in man by increasing urinary calcium (11) and in the rat where the feces represent the major pathway of calcium excretion, increased urinary calcium would affect calcium retention to a lesser extent. The interaction

TABLE 4

Calcium and ash of the whole body and femur and feed efficiency data

Criterion	Calcium, %			Buffering capacity [1]			EDTA, %		Lactose, %		SE
	0.19	0.48	0.78	0.46	0.54	0.76	0	0.2	0	12.0	
Body ash, g	5.69	6.90	6.65	6.99	6.28	6.08	6.82	6.01	6.39	6.50	0.09
Body calcium, g	1.56	1.98	1.96	1.98	1.81	1.71	1.93	1.74	1.79	1.88	0.03
Femur ash, mg	174	201	202	198	190	190	202	183	193	192	2.6
Femur ash,[2] %	62.3	66.0	66.7	65.9	64.6	64.6	65.8	64.2	64.9	65.1	0.2
Femur calcium, mg	57	69	73	69	66	64	69	63	64	68	1.4
Femur calcium,[2] %	20.6	22.7	23.9	23.0	22.4	21.8	22.7	22.0	21.8	22.9	0.2
Weight gain, g/6 weeks	117	132	103	125	119	108	125	110	117	117	2.4
Feed intake, g/6 weeks	591	592	530	594	565	555	582	560	571	572	7.5
Feed efficiency [3]	5.4	4.5	5.7	4.8	5.0	5.6	4.9	5.3	5.1	5.2	0.1

[1] Values represent BC 1, BC 2, and BC 3, respectively, as milliliters of 1 N HCl required to reduce pH of 5 g of the mixed diet by one pH unit.

[2] All bone data are on dry fat-free basis.

[3] Grams of feed per gram of weight gain.

between calcium level and dietary BC on calcium retention (table 3) showed that BC 1 and 0.48% calcium gave a total calcium retention value comparable to the one obtained with 0.78% calcium. Increasing calcium intake from 0.19 to 0.78% decreased percentage calcium retention by more than 50%, whereas the decrease from BC 1 to BC 3 was more than 20%.

Although calcium and ash content of the body and femur was increased ($P < 0.05$) by feeding the more acid (BC 1) diets (table 4), such an increase would be expected to be smaller as the fecal-to-urinary calcium ratio became greater. It is possible that the fecal-to-urinary calcium ratio reported for the rat (4, 7, 16) might represent a difference in the level of calcium intake or buffering capacity of the diets consumed. The BC 1 diets produced increased weight gain ($P < 0.01$) and increased feed intake ($P < 0.05$), in comparison with BC 3 diets, which resulted in improved feed efficiency ($P < 0.01$). The decreased dietary pH was reported to increase the rate of digesta acidification in the stomach and indirectly the rate of digestion and disappearance of nutrients from the stomach (14). The BC 1 diets gave the lowest gastric pH and highest duodenal pH.[6] These 2 factors would be expected to increase the stomach-emptying rate and could be responsible for the increased feed intake that resulted in an increased weight gain.

Lactose decreased ($P < 0.05$) total and percentage fecal calcium (fig. 1 and table 3) and increased calcium retention ($P < 0.05$). The effect of lactose on calcium utilization was reported (32) not to be on absorption but rather on calcium deposition, on the basis that lactose would affect the metabolic steps responsible for synthesis of the organic matrix at the ossification sites. From other evidence presented (34),[7] the beneficial effect of lactose was found to be completely confined to the absorption processes of calcium.

The interaction between lactose and calcium level on calcium retention (table 3)

[6] Ali, R. A. M., and J. L. Evans 1966 Effect of dietary calcium, buffering capacity, lactose and EDTA on pH of gastrointestinal contents, serum calcium and serum alkaline phosphatase activity in the growing rat. Federation Proc., 25: 367 (abstract).

[7] See footnote 5.

showed that the beneficial effect of feeding lactose was more pronounced when calcium supply in the diet was limited, especially when retention was expressed as percentage of calcium intake (table 3 and fig. 1). The interaction of lactose and dietary BC, however, showed a relationship between the effect of lactose on calcium retention and the acid-base value of the diet. The combination of BC 1 and lactose in the diet produced the highest calcium retention values whether expressed on the basis of total calcium retention or as the percentage of calcium intake (table 3 and fig. 1).

The increased ($P < 0.01$) calcium deposition in the bones was made evident by feeding lactose (table 4). Although lactose showed no main effect on both weight gain and feed intake, it interacted with dietary BC in increasing feed intake and feed efficiency. Both dietary BC and lactose showed direct effects on the pH pattern of the gastrointestinal tract which would affect the level of feed intake (14).[8]

The decreased calcium retention ($P < 0.05$) from feeding EDTA was the result of increased fecal calcium output ($P < 0.05$, table 3 and fig. 1). The effect was expected to be greater on urinary calcium excretion, especially with BC 3 diets, since it was reported that ingestion of bicarbonate after oral Ca-EDTA administration resulted in an enhanced urinary calcium output (35). Calcium retention was decreased most with EDTA in combination with BC 1 diets. Decreased body ash and calcium ($P < 0.05$) and femur ash and calcium ($P < 0.01$, table 4) resulted from the ingestion of a diet containing 0.2% EDTA. EDTA decreased weight gain ($P < 0.01$) and feed intake and efficiency but not significantly (table 4), and this might reflect the stress put on the animal by feeding EDTA.

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LITERATURE CITED

1. Fairbanks, B. W., and H. H. Mitchell 1936 The relation between calcium retention and the store of calcium in the body, with particular reference to the determination of calcium requirements. J. Nutr., *11:* 551.
2. Rottensten, K. V. 1938 The effect of body stores on the efficiency of calcium utilization. Biochem. J., *32:* 1285.
3. Forbes, R. M. 1964 Mineral utilization in the rat; effects of calcium, phosphorus, lactose and source of protein in zinc-deficient and in zinc-adequate diets. J. Nutr., *83:* 225.
4. Hansard, S. L., and M. P. Plumlee 1954 Effects of dietary calcium and phosphorus levels on the physiological behavior of calcium and phosphorus in the rat. J. Nutr., *54:* 17.
5. Davis, G. K. 1959 Effects of high calcium intakes on the absorption of other nutrients. Federation Proc., *18:* 1119.
6. Lanford, C. S., H. L. Campbell and H. C. Sherman 1941 Influence of different nutritional conditions upon the level of attainment in the normal increase of calcium in the growing body. J. Biol. Chem., *137:* 627.
7. 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. Nutr., *7:* 147.
8. 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. Nutr., *10:* 363.
9. Combs, G. E., T. H. Berry, H. D. Wallace and R. C. Crum, Jr. 1966 Levels and sources of vitamin D for pigs fed diets containing varying quantities of calcium. J. Animal Sci., *25:* 827.
10. Givens, M. H. 1918 Studies in calcium and magnesium metabolism. Further observations on the effect of acid and other dietary factors. J. Biol. Chem., *35:* 241.
11. Bogart, L. J., and E. E. Kirkpatrick 1922 Studies in inorganic metabolism. II. The effects of acid-forming and base-forming diets upon calcium metabolism. J. Biol. Chem., *54:* 375.
12. Shohl, A. T. 1937 The effect of the acid-base content of the diet upon the production and cure of rickets with special reference to citrate. J. Nutr., *14:* 69.
13. Thomas, J. E. 1957 Mechanics and regulation of gastric emptying. Physiol. Rev., *37:* 453.
14. Rogers, Q. R., and A. E. Harper 1966 Protein digestion: nutritional and metabolic consideration; effect of dietary pH. World Rev. Nutr. Diet., *6:* 250.
15. Hara, T. 1951–1952 Experimental studies on the utilization of calcium with special reference to the availability of calcium of bone meal. Japan. J. Nutr., *19:* 31.
16. Henry, K. M., and S. K. Kon 1939 Studies on the metabolism of calcium and phosphorus and on the availibility of these elements from milk and from inorganic sources. Biochem. J., *33:* 173.
17. Outhouse, J., J. Smith, L. Merritt and F. R. White 1937 A comparative study of the growth-promoting and bone-calcifying effects of several carbohydrates. J. Nutr., *14:* 579.

[8] See footnote 6.

18. Mills, R., H. Breiter, E. Kampster, B. McKey, M. Pickens and J. Outhouse 1940 The influence of lactose on calcium retention in children. J. Nutr., 20: 467.
19. Fournier, P. L., and Y. Dupuis 1962 Relation between absorption and utilization of calcium. Compt. Rend. Acad. Sci., 255: 1649.
20. Whittier, E. O., C. A. Cary and N. R. Ellis 1935 The effects of lactose on growth and longevity. J. Nutr., 9: 521.
21. Tomarelli, R. M., R. Hartz and F. W. Bernhart 1960 The effect of lactose feeding on the body fat of the rat. J. Nutr., 71: 221.
22. Kline, O. L., J. A. Keenan, C. A. Elvehjem and E. B. Hart 1932 Lactose in nutrition. J. Biol. Chem., 98: 121.
23. Hawkins, W. W., V. G. Leonard, J. E. Maxwell and K. S. Rastogi 1962 A study of the prolonged intake of small amounts of EDTA on the utilization of low dietary levels of calcium and iron by the rat. Can. J. Biochem. Physiol., 40: 391.
24. Clawson, A. J., J. T. Reid, B. E. Sheffy and J. P. Williams 1955 Use of chromic oxide in digestion studies with swine. J. Animal. Sci., 14: 700.
25. Johnson, D. E., W. E. Dinusson and D. W. Bolin 1964 Rate of passage of chromic oxide and composition of digesta along the alimentary tract of wethers. J. Animal Sci., 23: 499.
26. Czarnocki, J., I. R. Sibbold and E. V. Evans 1961 The determination of chromic oxide in samples of feed and excreta by acid digestion and spectrophotometry. Can. J. Animal Sci., 41: 167.
27. Carter, J. F., D. W. Bolin and D. Erickson 1960 The evaluation of forages by the agronomic "difference" method and the chromogen chromic oxide "indicator" technique. North Dakota Agricultural Experiment Station Tech. Bull. 426, State College Station, N. D.
28. Appleton, H. D., M. West, M Mandel and A. M. Sala 1959 The rapid determination of calcium in biological material. Clin. Chem., 5: 36.
29. Mori, K. 1959 Direct micro-complexometric analysis of calcium in biological materials. Arch. Biochem. Biophys., 83: 552.
30. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State College Press, Ames., pp. 329–393.
31. Henry, K. M., S. K. Kron, P. E. Todd, J. Toothill and D. H. Tomlin 1960 Calcium and phosphorus metabolism in the rat: effect of age on rate of adaptation to a low calcium intake. Acta Biochim. Pol., 7: 167.
32. Fournier, P. L. 1955 The relation between utilization of the structural carbohydrates and calcification. Compt. Rend. Acad. Sci., 240: 1364.
33. Fournier, P. L., Y. Dupuis, H. Susbielle, M. Allez and N. Tardy 1955 Mode of action of structural carbohydrates on calcium utilization. J. Physiol. (Paris), 47: 351.
34. Lengemann, F. W. 1959 The site of action of lactose in the enhancement of calcium utilization. J. Nutr., 69: 23.
35. Rubin, M 1963 The biological implications of alkaline earth chelation. In: The Transfer of Calcium and Strontium Across Biological Membranes, ed., R. H. Wasserman. Academic Press, New York.
36. National Research Council, Committee on Animal Nutrition 1962 Nutrient requirements of laboratory animals, pub. 990. National Academy of Sciences — National Research Council, Washington, D. C., pp. 51–80.

Accumulation and Elimination of Dietary Gossypol in the Organs of Rainbow Trout [1,2]

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ABSTRACT A study was made to determine the pattern of accumulation and elimination of dietary gossypol from various organs of rainbow trout. Young fingerling trout were fed purified diets containing gossypol at levels of 250 and 1000 ppm. Fish fed 1000 ppm gossypol for 18 months accumulated large amounts of gossypol in their tissue, with the highest concentration being in the liver and the lowest in the muscle tissue. After the fish had received a gossypol-free recovery diet for 10 weeks, these tissue levels were only partially depleted, with the free gossypol levels being lowered much more than the bound. The bound gossypol content of the liver was not significantly reduced over this 10-week period. Fish raised with a gossypol-free control diet for 9 months and then fed 1000 ppm dietary gossypol for 2 months accumulated much lower levels of free gossypol in their organs, but levels of bound gossypol approached those of fish fed gossypol for 18 months. Fish fed 250 ppm dietary gossypol for 12 months accumulated lower concentrations of gossypol in their organs. In all cases, the liver accumulated the highest concentration of gossypol.

Gossypol is a yellow pigment formed in the cotton plant and is present in varying amounts in processed cottonseed meal. When fed in sufficient quantities, this compound has been shown to be toxic to a large number of nonruminant animals (1–6). In some cottonseed meals, gossypol may be present in sufficient quantities to cause this toxicity, and its use in animal feeding is therefore limited.

The toxicity of this compound has been attributed by Menual (7) to its inhibition of the conversion of oxyhemoglobin to hemoglobin in the blood, and also to hemolysis of erythrocytes. The resulting stress on respiratory and circulatory organs commonly produces edema of the lungs, hemolytic anemia, and cardiac failure. More general symptoms are anorexia, growth depression, and finally death. The accumulation of ingested gossypol in body tissues was demonstrated recently with swine, where the buildup was particularly high in the liver (8–9). In general, the organs that accumulated large amounts of gossypol were the ones that Menual had found to have severe histological damage in cases of toxicity.

Since trout rations are high in protein, 35 to 50%, any toxic compound contaminating the protein source might reach dangerous levels in the diet. A diet containing 25% cottonseed meal could contain from 100 to 250 ppm free gossypol. Therefore the present study was undertaken to study the accumulation and elimination of gossypol in rainbow trout.

MATERIALS AND METHODS

Fish used in this experiment were Mt. Shasta strain rainbow trout (*Salmo gairdnerii*). Eggs from the parent fish were hatched in our laboratory, and reared in well-water at a constant temperature of 11.5°. The experimental diets [3] were fed to duplicate lots of trout held in 122-cm circular fiberglass tanks. The fish received from one to three feedings per day depending on their size, and were fed 90% of the ad libitum amount. This was approximately 4% of body weight when they were small to 2% as the feeding period progressed. Fish receiving 1000 ppm gossypol consumed only one-half as much as control fish.

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TABLE 1

Composition of experimental trout diets

	Composition [1]	
	Diet 1 (control)	Diet 2 (control)
Casein, vitamin-free	49.4	49.4
Gelatin	8.7	8.7
Dextrin	15.6	15.6
Corn oil	14.0	5.0
Salmon oil	—	5.0
Salt mix no. 2 USP XIII	4.0	4.0
Calcium carbonate	0.9	0.9
Carboxymethylcellulose	1.3	1.3
Cellulose [2]	2.5	6.5
Vitamin mix [3]	2.0	2.0
Choline chloride	1.0	1.0
α-Tocopheryl acetate (110 IU/g)	0.6	0.6

[1] Diet 1 is control plus 1000 ppm gossypol-acetic acid added in the lipid premix. Diet 2 contained 250 ppm gossypol added in the same way.

[2] Alphacel, Corn Products Company, Argo, Illinois.

[3] Supplied following levels of vitamins and preservatives: (mg/kg diet) thiamine, 64; riboflavin, 144; niacinamide, 512; biotin, 1.6; Ca D-pantothenate, 288; pyridoxine, 48; folic acid, 19.2; menadione, 16; cobalamine, 0.159; *i*-inositol (*meso*), 2500; ascorbic acid, 1200; *p*-aminobenzoic acid, 400; butylated hydroxy anisole, 15; vitamin D, 4000 IU; vitamin A, 216,000 IU; butylated hydroxy toluene, 15.

In the first of 3 experiments, duplicate lots of 150 newly hatched fry received 1000 ppm gossypol acetic acid (diet 1, see table 1) for 18 months. Samples were taken at this time for gossypol analysis and the remaining fish were fed control diet 1 for 10 weeks. Random samples were taken after the fish had received the control diet for one, three, five and ten weeks.

In experiment 2, 50 nine-month-old control-fed fish weighing approximately 150 g each were fed the 1000-ppm gossypol-acetic acid diet (diet 1) for 8 weeks. A random sample was taken and the remaining fish were returned to the control diet for 10 weeks at which time a final sample was taken for gossypol analysis.

Experiment 3 consisted of feeding 250 ppm gossypol-acetic acid in the control diet (diet 2) for 12 months and determining the accumulation of gossypol in the tissues of fish selected at random. Three-hundred 30-day-old fry were fed this experimental diet. The experimental diet was changed as shown in table 1 for experiment 3 because the addition of salmon oil was found to increase the growth rate and reduce mortality (12).

The organs from each sample fish were removed, placed in sealed jars, and stored at $-28°$ until analyzed. In some instances, organs such as the heart and spleen were so small that the use of pooled samples was necessary. Each organ or group of organs was weighed, and gossypol determined by the procedure of Smith (11). In this procedure, free gossypol is defined as that gossypol which can be extracted from the tissue with ethanol:ether 5:2 (v/v). Bound gossypol is that which remains in the tissue after the solvent extraction and is removed by reacting the tissue with aniline and then extracting it with hexane. Quantitative analysis is effected by measuring the absorbance of the aniline condensation product of the extracted gossypol, and comparing it to a standard curve.

RESULTS

Feeding the diet containing 1000 ppm gossypol (diet 1) for 18 months resulted in large accumulations of both free and bound gossypol in the organs of rainbow trout (table 2). This accumulation was particularly high in the liver and, to a somewhat lesser extent, in the spleen and kidney. The lowest concentration of gossypol was noted in the muscle tissue.

After feeding this group of fish a gossypol-free recovery diet for 10 weeks, all the organs sampled contained significantly reduced ($P < 0.01$) concentrations of free gossypol. Bound gossypol, however, was reduced only in the gastrointestinal tract, and increased considerably in the pooled samples of heart and spleen tissue. The bound gossypol in the liver increased significantly ($P < 0.01$) after one week of the control, and then decreased to the original level. Over the 10-week period, therefore, there was no net change in bound gossypol in the liver. A similar effect was observed in the kidney tissue.

The fish in this group accepted the diet well, but had a much lower feed consumption and approximately one-half the growth rate of the controls. Fish fed higher levels of gossypol (2000 ppm) did not accept the diet.

Fish fed diet 1 for a 2-month period (table 3) accumulated smaller concentrations of gossypol in their tissue. Again the greatest accumulation was in the liver, whereas the muscle contained so little gossypol it could not be quantitatively assayed. When these fish were fed a gossypol-free

TABLE 2

Gossypol levels in tissues from fish fed 1000 ppm gossypol-acetic acid 18 months then the control diet for a 10-week period (exp. 1)

Tissue	n_o[1]	Gossypol	Weeks fed gossypol-free diet					L.S.D.	
			0	1	3	5	10	0.05	0.01
			μg gossypol/g tissue						
Liver	4.59	free	358	415	232	266	167	89.3	122.4
	4.59	bound	202	319	220	282	203	77.6	106.3
Kidney	2.93	free	302	291	161	181	126	74.0	105.3
	2.75	bound	77	196	120	151	121	56.5	81.2
Muscle	3.55	free	10.7	9.8	4.5	—	—	2.7	3.9
	3.43	bound	2.9	3.7	2.1	2.0	—	1.7	2.4
GI tract	3.38	free	73	40	14	21	16	13.6	19.0
	3.38	bound	28	21	15	19	16	12.0	16.8
Heart	P(5)[2]	free	373	125	70	51	30	—	—
	P(5)	bound	18	54	21	25	34	—	—
Spleen	P(5)	free	392	327	144	177	121	—	—
	P(5)	bound	69	216	166	192	215	—	—

[1] n_o is the pooled replication, calculated by the formula $n_o = \frac{1}{k-1} n - \frac{n^2}{n}$ where k = the number of treatments, and n = the number of replications per treatment (13).

[2] Denotes a pooled sample of 5 organs.

TABLE 3

Gossypol content of tissues from rainbow trout fed diet 1 (1000 ppm gossypol) for 2 months and diet 2 (250 ppm gossypol) for 12 months [1]

Tissue	Gossypol	Exp. 2		Exp. 3
		Diet 1	10-week recovery [2]	Diet 2
		μg gossypol/g tissue		
Liver	free	207	83	83
	bound	109	100	94
Kidney	free	41	40	45
	bound	88	23	73
Muscle	free	—	—	—
	bound	—	—	—
GI tract	free	27	11	25
	bound	12	5	10
Heart	free	39	22	32
	bound	26	16	18
Spleen	free	108	82	49
	bound	53	47	44

[1] Values represent pooled samples of 5 organs each.
[2] Fish fed diet 1 for 2 months were then fed a gossypol-free control diet.

diet for 10 weeks, there was a general reduction in both free and bound gossypol in all tissues except the liver and spleen, which showed no change in bound gossypol content. The fish in this group showed no adverse effects from the high level of gossypol fed for the 2-month period. Food consumption was lower than that of the control.

In experiment 3, fish fed diet 2 (250 ppm gossypol) for a 12-month period showed no growth depression as in experiment 1 in which 1000 ppm gossypol were fed. The gossypol levels of their organs were generally comparable to those of the fish fed diet 1 for 2 months with lower levels of free gossypol in the liver and spleen (table 3). As in experiments 1 and 2, the highest concentration of gossypol was in the liver.

DISCUSSION

The accumulation of dietary gossypol in the tissues of rainbow trout was similar to that reported in swine by Smith and Clawson (9), and Sharma et al. (12). The highly vascular organs showed the highest concentrations, with elevated levels of free gossypol accumulating in these organs (liver, kidney, spleen and heart) with an increased gossypol intake. In contrast, the bound gossypol levels were similar for all three gossypol-fed groups in all tissues except the liver. This was unexpected as either the levels of dietary gossypol, time fed the experimental diet, or the age of the fish was different in each feeding trial.

The levels of bound gossypol in the tissues of fish fed 250 ppm for 12 months were one-half or more of those in fish receiving 1000 ppm for 18 months. This

indicates the possibility of a slow buildup of gossypol for extended periods of time. The importance in determining the long-range effects of ingested gossypol was demonstrated by Sharma et al. (12) who reported that low dietary protein levels enhanced the accumulation of gossypol in the tissues of swine. In this respect, it should be pointed out that adequate levels of protein were fed in the experimental diets used in this study (table 1).

The increase in bound gossypol levels in the tissues of fish removed from the high gossypol diet after an 18-month period cannot be explained at present. There was an apparent shift of accumulated gossypol from muscle, stomach, intestines and heart to the liver, spleen and kidney immediately after the fish were fed a gossypol-free diet. At the same time there was an increase in the bound gossypol levels of all tissues except muscle and gastrointestinal tract. Free gossypol levels in all tissues dropped significantly during the 10-week period, whereas the levels of bound gossypol increased or showed little change. Although it appears unlikely that "free" gossypol exists in a totally free state in the tissue, the difference in the type, or types, of binding which distinguished free from bound gossypol appears to be an important factor in the elimination of gossypol.

The presence of two reactive carbonyl groups on the gossypol molecule as well as the multiplicity of nucleophiles available for reaction in vivo makes possible a wide variety of "bound" compounds. These compounds probably vary widely in their motility within the body, and make the mechanism of their elimination extremely complex.

The high levels of gossypol noted in the liver tissue appear to indicate that this is the main organ responsible for elimination of these compounds, and that this elimination takes place with considerable difficulty.

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LITERATURE CITED

1. Withers, W. A., and F. E. Carruth 1915 Gossypol, the toxic substance in cottenseed meal. J. Agr. Res., 5: 281.
2. Schwartz, E. W., and C. L. Alsberg 1924 Pharmacological action of gossypol. J. Agr. Res., 28: 191.
3. Eagle, E. 1950 Effects of repeated doses of gossypol on the dog. Arch. Biochem., 26: 68.
4. Harms, W. S., and K. T. Holley 1951 Hypoprothrombinemia induced by gossypol. Proc. Soc. Exp. Biol. Med., 77: 297.
5. Couch, J. R., W. Y. Chang and C. M. Lyman 1955 The effect of free gossypol on chick growth. Poultry. Sci., 34: 178.
6. Rigdon, R. H., G. Cross, T. M. Ferguson and J. R. Couch 1958 Effects of gossypol on young chicks with the production of a ceroid-like pigment. Arch. Pathol., 65: 228.
7. Menual, P. 1923 The physiological effect of gossypol. J. Agr. Res., 26: 233.
8. Clawson, A. J., F. H. Smith and E. R. Barrick 1962 Accumulation of gossypol in livers and factors influencing toxicity of injected gossypol. J. Animal Sci., 21: 911.
9. Smith, F. H., and A. J. Clawson 1965 Effects of diet on accumulation of gossypol in the organs of swine. J. Nutr., 87: 317.
10. Lee, D. J., J. N. Roehm, T. C. Yu and R. O. Sinnhuber 1967 Effects of ω3 fatty acids on the growth rate of rainbow trout (*Salmo gairdnerii*). J. Nutr., 92: 93.
11. Smith, F. H. 1965 Determination of free and bound gossypol in swine tissues. J. Amer. Oil Chem. Soc., 42: 145.
12. Sharam, M. P., F. H. Smith and A. J. Clawson 1966 Effects of levels of protein and gossypol, and length of feeding period on the accumulation of gossypol in tissues of swine. J. Nutr., 88: 434.
13. Li, J. C. R. 1957 Introduction to Statistical Inference. Edwards Brothers, Ann Arbor, Michigan, p. 178.

Nitrogenous Factors Affecting the Adequacy of Rice to Meet the Protein Requirements of Human Adults [1]

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ABSTRACT A study was made to determine the first-limiting nitrogenous component in rice protein for nitrogen retention in human adults. Six subjects fed diets providing 1) 6 g nitrogen from rice or 6 g nitrogen from rice supplemeted with 2) 2 g amino acid nitrogen combined in the rice pattern, 3) 2 g nonspecific nitrogen, and 4) 6 g nonspecific nitrogen, achieved average nitrogen balances of − 0.01, + 0.23, − 0.13, and + 0.25 g/day, respectively. The study was 43 days in length and included three 10-day and one 5-day experimental periods. A second study was made to determine the first-limiting essential amino acid in rice protein for nitrogen retention when the diet contained adequate amounts of total nitrogen (12 g/day). The study was 55 days in length with experimental periods of 5 days. In the first part of the experiment, reducing the amount of rice to provide from 6 to 2 g nitrogen/day resulted in highly significant differences in nitrogen balances of 6 adult subjects. Mean nitrogen balances were: + 0.45 (6 g rice N), − 0.31 (4 g rice N), − 0.70 (3 g rice N), and − 1.13 g/day (2 g rice N). Subsequently, various essential amino acids or amino acid combinations, suspected as being limited in rice protein, were tested in subjects fed minimal levels of rice nitrogen and a high level of total nitrogen (12 g/day). Mean nitrogen balances of subjects fed rice supplemented with amino acids as provided by 0.5 g rice nitrogen were + 0.15 (EAA plus cystine and tyrosine), + 0.26 (lysine plus threonine), + 0.14 (lysine), − 0.32 (threonine), and − 0.03 g/day (methionine plus cystine). Nitrogen retentions were significantly higher in response to the lysine-containing supplements than to those containing threonine alone or methionine-cystine, indicating that lysine is the first-limiting amino acid in rice protein for supporting nitrogen retention when the diet provides a high level of total nitrogen.

A comparison of rice protein with high quality proteins indicates a lower content of nitrogen and of certain essential amino acids in rice proteins. A common approach used in improving the nutritive value of rice protein has been to supplement with purified amino acids (1, 2) or with high quality animal proteins (3–5).

Recent reports have indicated that the total amount of dietary nitrogen as well as the amount of essential amino acids may be important in determining the adequacy of the dietary protein. Snyderman et al. (6) demonstrated that growth of infants receiving inadequate amounts of milk protein could be restored and nitrogen retention improved by the addition of nonessential nitrogen from either glycine or urea. Results of Kies et al.[3] indicated that the addition of nitrogen from a variety of sources improved the nitrogen balance response of adult men fed inadequate amounts of corn protein.

The objectives of the present project were to determine the order in which nitrogenous factors limit the usefulness of rice protein for human adults and to determine the first-limiting essential amino acid in rice protein when the diet provides an adequate amount of total nitrogen.

EXPERIMENTAL

Two separate metabolism studies were conducted. The first study was designed to compare the adequacy for support of nitro-

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[3] Kies, C., H. M. Fox and E. Williams 1965 Effect of quantitative-qualitative variations in "non-specific" nitrogen supplementation of several dietary proteins. Proc. Soc. Exp. Biol., 25: 300 (abstract).

TABLE 1

Diet plans

Diet type	Time fed	Dietary nitrogen, sources and amount			
		Rice	Glycine [1] + diammonium citrate	Purified amino acids [2]	Total N intake [3]
	days	*g N/subject/day*			
		Study 1 [4]			
Depletion	3	1.1	0	0	1.7
Adjustment	5	6.0	0	0	6.6
Exp. 1	10	6.0	0	0	6.6
Exp. 2	10	6.0	2.0	0	8.6
Exp. 3	5	6.0	0.65	1.35 (10 AA)	8.6
Exp. 4	10	6.0	6.0	0	12.6
		Study 2 [5]			
Depletion	2	1.1	0	0	1.8
Adjustment	3	6.0	6.0	0	12.7
Exp. 1	5	2.0	10.0	0	12.7
Exp. 2	5	3.0	9.0	0	12.7
Exp. 3	5	4.0	8.0	0	12.7
Exp. 4	5	6.0	6.0	0	12.7
Exp. 5	5	+ [6]	+ [6]	0	12.7
Exp. 6	5	+ [6]	+ [6]	10 AA	12.7
Exp. 7	5	+ [6]	+ [6]	lysine	12.7
Exp. 8	5	+ [6]	+ [6]	threonine	12.7
Exp. 9	5	+ [6]	+ [6]	threonine + lysine	12.7
Exp. 10	5	+ [6]	+ [6]	methionine + cystine	12.7

[1] Glycine and diammonium citrate in isonitrogenous amounts were used as the dietary supplementary source of nonspecific nitrogen.

[2] Purified amino acids were fed in the amounts and proportions present in 2.0 g rice nitrogen (essential amino acids plus cystine and tyrosine) in diet 3, study 1 or in 5.0 g rice nitrogen as specified in diets 5 through 10, study 2.

[3] Total nitrogen intake figures include 0.6 to 0.7 g nitrogen furnished by basal diet. This consisted of the following items (g/subject/day) in study 1: tomato juice, 80; cabbage, 50; green beans, 100; pears or applesauce, 100; beef boullion, 14; coffee or tea, varied. In study 2: tomato juice, 80; cabbage, 50; green beans, 100; peaches, 100; pineapple, 100; beef bouillon, 14; coffee or tea, varied; jelly, varied. A multiple vitamin supplement provided the following: vitamin A, 5000 IU; vitamin D, 500 IU; thiamine, 2 mg; riboflavin, 2.5 mg; ascorbic acid, 50 mg; pyridoxine, 1 mg; vitamin B_{12}, 1 μg; niacinamide, 20 mg; and pantothenic acid, 1 mg per subject per day; and 4.3 mg of mineral supplement provided (g/subject/day): Ca, 0.66; P, 0.36; Mg, 0.24; Fe, 0.02; Cu, 0.002; I, 0.0002; Mn, 0.002; and Zn, 0.001.

[4] Experimental periods during which experimental diets 1 through 4, study 1, were fed were arranged at random; however, within this randomization, the period in which diet 3 was fed always directly followed that in which diet 2 was given in time sequence.

[5] Experimental periods during which experimental diets 1 through 4, study 2, were fed were arranged at random. These periods were followed by a period during which subjects received diet 5 which provided 5 g rice nitrogen for subjects T, W, and P; 2 and 3.5 g rice nitrogen for subjects S and X. (These amounts were selected on the basis of results of the four earlier experimental periods as probably being midway between adequate and inadequate amounts.) Experimental periods during which diets 6 through 10 were fed were arranged at random for each subject. During these periods, rice provided 5.0 g nitrogen/day for subject T, 4.0 g nitrogen/day for subjects, S, P and Z, 3.0 g nitrogen/day for subject X, and 2.5 g nitrogen/day for subject W (amounts as determined in earlier periods as probably being just slightly inadequate). The glycine-diammonium citrate mixture maintained total nitrogen intake constant at 12.7 g/day.

[6] Varied with subject.

gen balance of diets providing protein almost entirely from rice with diets in which rice was supplemented with amino acids or with a low or high level of glycine and diammonium citrate, designated as nonspecific nitrogen. The purpose of the second study was to determine the first-limiting essential amino acid in rice protein for supporting nitrogen balance when total nitrogen intake was maintained at a high level by adding nonspecific nitrogen to the diet.

The experimental diet plans for the 2 studies are shown in table 1. Both studies included a period of low nitrogen feeding to deplete subjects of easily lost protein reserves and a period of adjustment to the experimental diet. The experimental periods were 5 or 10 days in length, 5 days when successive diets were of the same nitrogen content and 10 days when the nitrogen content varied from period to period. Subjects were assigned to the various experimental treatments in a random manner.

Subjects. The University of Nebraska students who were subjects for the studies are described in table 2. All were in

good health as revealed by a physician's examination preceding and following the experimental periods and maintained their regular school activities (study 1) or were engaged in laboratory work during the time of the experiment (study 2).

Diets.[4] The depletion diet fed at the beginning of both experiments (table 1) provided 1.7 to 1.8 g nitrogen daily.

The experimental diets used in study 1 (table 1) provided 6 g nitrogen from rice and approximately 0.6 g nitrogen from natural foods in the diet. The amount of rice fed remained constant throughout the experiment, 551 g daily. Rice was prepared by steaming and equal amounts were served at each of 3 meals. Nitrogenous supplements consisted of an amino acid mixture providing essential and nonessential amino acids combined in the rice pattern and an isonitrogenous mixture of glycine and diammonium citrate. The amino acid mixture was fed to provide amounts of amino acids present in 2.0 g rice nitrogen and glycine and diammonium citrate were added so that the total nitrogen content of the supplement was 2.0 g. In periods when nonspecific nitrogen was used as a supplement, glycine and diammonium citrate were combined isonitrogenously and made into solution. The solution, providing either 2.0 or 6.0 g nitrogen, was flavored with dextrose and lemon juice and served in equal amounts at each meal.

Experimental diets used in study 2 provided 12.7 g nitrogen daily, 2.0 to 6.0 g from rice, 6.0 to 10.0 g from a mixture of glycine and diammonium citrate and 0.7 g from natural foods. The five amino acid supplements used in the second part of the experiment were fed to provide amounts of the particular amino acid(s) present in 0.5 g rice nitrogen. Each supplement was fed to provide a total of 0.5 g nitrogen daily, the nitrogen content of the supplements being equalized by additions of glycine and diammonium citrate.

Dietary fat made up 20% of the total caloric intake for individual subjects in each study. In each case, one-half of the subjects received corn oil as the source of fat and the others, butterfat. Calories were adjusted to maintain constant body weight by appropriate adjustments in dextrose and fat intake. Experimental diets and depletion diets used in both studies also included low nitrogen fruits and vegetables, vitamins and mineral supplements as described in table 2.

Analyses. Complete collections of urine and feces were made throughout both studies. Total nitrogen was determined on 24-hour samples of urine, 5-day fecal composites, rice, natural foods, amino acid

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TABLE 2

Data on subjects

Subject code	Sex	Nationality	Height	Age	Weight	
					Initial	Final
			cm	*years*	*kg*	*kg*
			Study 1			
A	M	Chinese	165	25	65.9	65.5
B	M	American	173	23	74.1	74.0
C	M	Turkish	173	31	67.5	66.8
D	F	Chinese	157	34	62.3	62.7
E	F	American	183	21	78.2	78.1
F	M	Chinese	188	39	74.1	75.0
			Study 2			
S	M	American	183	20	90.0	90.3
T	M	American	185	21	87.5	86.8
W	M	American	170	27	70.0	70.0
X	F	American	183	22	79.0	80.4
P	M	American	152	21	64.7	65.0
Z	M	American	160	21	96.0	97.7

TABLE 3

Mean nitrogen balances of subjects fed unsupplemented and supplemented rice diets

Subject	6 g N (rice)	6 g N (rice) + 2 g N (NSN) [1]	6 g N (rice) + 2 g N (AA) [2]	6 g N (rice) + 6 g N (NSN) [1]
			g/day	
A	−0.50	−0.31	0.40	0.49
B	0.29	−0.30	0.15	0.47
C	−0.02	−0.51	−0.12	−0.09
D	0.32	0.38	−0.03	0.08
E	−0.14	−0.23	0.28	0.00
F	−0.03	0.19	0.67	0.52
Mean	−0.01	−0.13	0.23	0.25

[1] NSN indicates nonspecific nitrogen; in this case, nitrogen provided by an isonitrogenous mixture of glycine and diammonium citrate.
[2] AA indicates amino acids; in this case, the essential amino acids plus cystine and tyrosine as present in 2.0 g rice nitrogen plus glycine and diammonium citrate so as to provide 2.0 g of nitrogen.

supplements and the glycine-diammonium citrate solution by the boric acid modification of the Kjeldahl method according to Scales and Harrison (7).

RESULTS

Individual and mean nitrogen balances of subjects fed diets providing 6 g rice nitrogen and 6 g rice nitrogen supplemented with 2 g nonspecific nitrogen, 2 g amino acid nitrogen and 6 g nonspecific nitrogen are shown in table 3. Mean nitrogen balances achieved with the experimental diets were: − 0.01 (6 g rice N), − 0.13 (6 g rice N, 2 g nonspecific N), + 0.23 (6 g rice N, 2 g amino acid N), and + 0.25 g/day (6 g rice N, 6 g nonspecific N).

While nitrogen balance tended to improve when a low level of amino acid nitrogen or a high level of nonspecific nitrogen was added to a rice diet, no improvement was noted when a low level of nonspecific nitrogen was used as the supplement. Although the increases in nitrogen balance were not statistically significant, three of the six subjects showed a distinct improvement in nitrogen retention in response to the 2 types of supplements, 2 g N (AA) or 6 g nonspecific N.

Nitrogen balances of individual subjects and the means for all subjects in study 2 are shown in figure 1. Nitrogen retention improved progressively as the level of rice nitrogen was increased, and the total nitrogen intake was held constant by appropriate adjustments in the level of nonspecific nitrogen. Mean nitrogen balances were − 1.13, − 0.70, − 0.31 and + 0.45 g/day when diets provided 2, 3, 4 and 6 g rice nitrogen, respectively. Statistical evaluation indicated highly significant differences between dietary treatments, with the exception of the diets providing 2 and 3 g rice nitrogen. Individual subjects showed a consistent response to the various levels of rice in the diet.

Mean nitrogen balances for subjects in the second part of study 2 fed an individually determined subotpimal level of rice nitrogen, a high level of total nitrogen, and supplementary nitrogen from one or more amino acids in amounts present in 0.5 g rice nitrogen were: − 0.38 (unsupple-

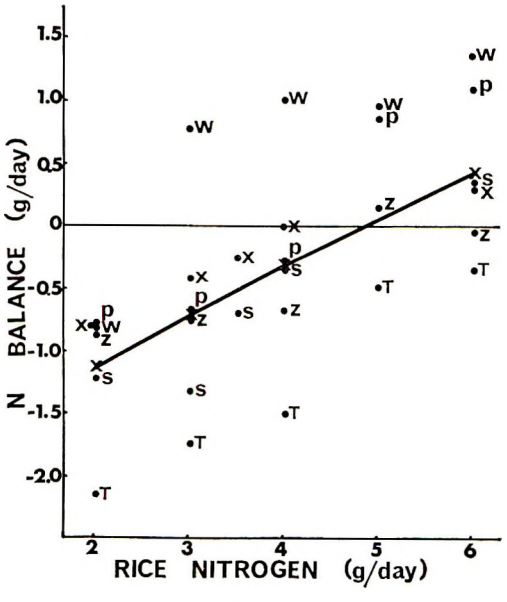

Fig. 1 Nitrogen balances of subjects fed different amounts of rice.

TABLE 4
Effect of supplementing inadequate rice diets with selected purified amino acids on nitrogen balances of adult men

Subject	Unsupplemented rice [2]	Mean N balances [1] of men fed rice supplemented [3] with				
		AA	Lysine	Threonine	Threonine + lysine	Methionine + cystine
	g/day	*g/day*	*g/day*	*g/day*	*g/day*	*g/day*
S	−0.35	−0.11	0.19	−0.26	−0.04	−0.15
T	−0.48	−0.04	0.20	−0.81	0.30	−0.15
W	−0.03	0.50	0.43	0.04	0.35	0.36
X	−0.43	−0.10	−0.41	−0.66	0.10	−0.43
P	−0.30	0.45	0.23	−0.03	0.70	0.71
Z	−0.68	0.20	0.16	−0.21	0.15	−0.51
Mean	−0.38	0.15	0.14	−0.32	0.26	−0.03

[1] Mean of the 5 values for each of the 5 days composing each experimental period.
[2] Level of rice nitrogen individualized to produce a slightly negative nitrogen balance (2.5 to 5.0 g rice nitrogen).
[3] Purified amino acid supplements were fed in the amounts and proportions as present in 0.5 g rice nitrogen. AA indicates the eight essential amino acids plus cystine and tyrosine.

mented diet), + 0.15 (10 amino acids), + 0.14 (lysine), −0.32 (threonine), + 0.26 (lysine and threonine), and − 0.03 g/day (methionine and cystine) as shown in table 4. All of the amino acid supplements, except threonine, improved nitrogen balance significantly over that observed with the unsupplemented diet. Nitrogen retention was improved by adding lysine-containing supplements to the rice ($P < 0.01$) and by adding methionine and cystine ($P < 0.05$). However, nitrogen retention was significantly higher in response to the lysine-threonine than to the methionine-cystine supplement. The results suggest that lysine is the first-limiting essential amino acid in rice protein for the support of nitrogen balance in adult human subjects.

DISCUSSION

Attempts to delineate the effect of kind and amount of dietary nitrogen on the nitrogen balance response of subjects fed diets providing protein primarily from rice may be compared with similar studies using corn as the dietary protein (8, 9). That rice may be a more adequate source of protein than corn is indicated by a comparison of the mean nitrogen balances of subjects fed 6 g nitrogen from either of these cereal proteins, − 0.50 g/day (corn) versus − 0.01 g/day (rice). At this level of nitrogen intake, rice contains larger quantities of both lysine and tryptophan than corn, which probably explains the somewhat higher nitrogen retention observed when rice was the source of protein.

Although small additions of nitrogen from either amino acids or nonspecific nitrogen sources brought about a marked improvement in nitrogen balance of subjects fed the corn diets, nitrogen balances of subjects fed rice was increased to a smaller extent and the improvement occurred only when small amounts of amino acid nitrogen or large amounts of nonspecific nitrogen were used as supplements.

Kies et al. (9) demonstrated that nitrogen balances of subjects fed suboptimal amounts of corn protein increased progressively as the level of nonspecific dietary nitrogen was increased. In attempting to explain the improvement in nitrogen balance induced by feeding high levels of nonspecific nitrogen, these workers speculated that nonessential nitrogen may enhance the absorption of essential amino acids (10). Applying this line of reasoning to data from the present study would offer a reasonable explanation for the similar effects on nitrogen retention observed when small amounts of amino acid nitrogen or large amounts of nonspecific nitrogen were added to the rice diet.

Although the results of the first study indicated that nitrogen balance was not improved with low level nonspecific nitrogen supplementation, the results of the second study demonstrated that with a high level of nonspecific nitrogen supplementation, the amount of rice needed to support nitrogen equilibrium could be lowered appreciably. This is in agreement with the

observations of Synderman et al. (6) and Kies et al. (8) that nitrogen is more limiting than essential amino acids when a single source of food is used to supply the nitrogenous needs of growing children or adult men. This observation implies that the quantities of essential amino acids needed by humans is dependent, at least in part, on the total nitrogen content of the diet. This may be an important consideration in interpreting the amino acid requirements of humans as determined by various investigators. Thus, Snyderman et al. (6) have suggested that the requirements for men as determined by Rose tend to be minimal since large amounts of nonessential nitrogen were fed, whereas the infant requirements determined by Snyderman and others (11) tend to be maximal since the diet provided amino acids in the milk pattern and no supplementary sources of nonspecific nitrogen.

Systematic reduction in essential amino acid content of the diet while maintaining total nitrogen constant at a high level appears to be a satisfactory method for demonstrating a need for the most limiting amino acid. Subsequent supplementation of the diet with possible limiting essential amino acids showed a clear-cut differentiation in response favoring lysine as the first-limiting essential amino acid in rice protein. Use of this method to study essential amino acid limitations in corn protein also gave definitive results (10). The corn and rice studies conducted in this laboratory plus the work of Synderman et al. (6) with milk protein represent the only attempts to measure essential amino acid limitations of food proteins in this manner.

LITERATURE CITED

1. Pecora, L. J., and J. M. Hundley 1951 Nutritional improvement of white polished rice by the addition of lysine and threonine. J. Nutr., *44:* 101.
2. Rosenberg, H. S., and R. Culik 1957 The improvement of the protein quality of white rice by lysine supplementation. J. Nutr., *63:* 477.
3. Deshpande, P. D., A. E. Harper, F. Quiros-Perez and C. A. Elvehjem 1955 Further observations on the improvement of polished rice with protein and amino acid supplements. J. Nutr., *57:* 415.
4. Kik, M. C. 1960 Further studies on the nutritional improvement of rice. J. Agr. Food Chem., *8:* 380.
5. Kik, M. C. 1962 Nutritive value of chicken meat and its value in supplementing rice protein. J. Agr. Food Chem., *10:* 59.
6. Snyderman, S. E., L. E. Holt, Jr., J. Dancis, E. Roitman, A. Boyer and M. E. Balis 1962 "Unessential" nitrogen: a limiting factor for human growth. J. Nutr., *78:* 57.
7. Scales, F. M., and A. P. Harrison 1920 Boric acid modification of the Kjeldahl method for crop and soil analysis. J. Ind. Eng. Chem., *12:* 350.
8. Kies, C., E. Williams and H. M. Fox 1965 Determination of first limiting nitrogenous factor in corn protein for nitrogen retention in human adults. J. Nutr., *86:* 350.
9. Kies, C., E. Williams and H. M. Fox 1965 Effect of "non-specific" nitrogen intake on adequacy of cereal proteins for nitrogen retention in human adults. J. Nutr., *86:* 357.
10. Kies, C., H. M. Fox and E. R. Williams 1967 Effect of non-specific nitrogen supplementation on minimum corn protein requirement and first limiting amino acid for adult men. J. Nutr., *92:* 377.
11. Snyderman, S. E., P. M. Norton, D. I. Fowler and L. E. Holt, Jr. 1959 Essential amino acid requirements of infants: lysine. J. Dis. Child., *97:* 175.

Plasma Lipids in Maternal and Fetal Rabbits Fed Stock and Peanut Oil-Cholesterol Diets [1,2]

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ABSTRACT In 2 groups of pregnant rabbits, one fed a stock diet and the other fed a peanut oil-cholesterol diet, the maternal and fetal plasma levels of phospholipids, triglycerides, ester and free cholesterol were determined. The relative percentage of fatty acid composition of the phospholipid, nonesterified fatty acid, triglyceride and cholesterol ester fractions were also studied. The changes in maternal plasma lipid levels and fatty acid composition correlated well with the amount of fat and the fatty acid composition of the diets consumed by the 2 groups. The only significant change in the level of these plasma lipids in the fetus of the 2 groups was a small increase in the cholesterol ester level of the fat-cholesterol diet group. There were several alterations in the fatty acid composition of the fetal plasma lipids between the two dietary groups. Nearly one half of these were correlated with changes in the diets consumed by the maternal rabbits. Changes in fetal plasma fatty acid composition that correlated with the diets consumed were at least partly explained on a placental transport basis. The changes in fetal fatty acid composition that were not correlated with the diet were probably due to indirect dietary-induced changes in fetal lipid metabolism, possibly through multiple step pathways.

The effect of high fat- and cholesterol-containing diets on the plasma lipids in the nonpregnant rabbit has been studied by several investigators (1, 2) but the effect of fat and cholesterol feeding during pregnancy on maternal and fetal plasma lipids has not been so thoroughly investigated. By feeding a diet supplemented with approximately 1 g/day of cholesterol to pregnant rabbits Popjak (3) was able to obtain marked increases in maternal plasma lipids; however, he stated that it was impossible to influence fetal plasma lipid values, except to a very limited extent, by cholesterol feeding of the mothers. In his study Popjak (3) measured total fatty acids, nonphospholipid fatty acids, neutral fat, phospholipids, free and total cholesterol.

The present study was conducted to investigate certain aspects of the placental transport of lipids by comparing maternal and fetal plasma lipid levels and fatty acid composition in 2 groups of rabbits fed diets differing in amounts of fat, cholesterol, and fatty acid composition. The levels of phospholipid, triglyceride, cholesterol ester and free cholesterol was measured in the plasma of maternal and fetal rabbits. The relative percentages of fatty acids were determined in the phospholipid, nonesterified fatty acid, triglyceride and cholesterol ester fractions of the maternal and fetal plasma by thin-layer and gas-phase chromatography.

MATERIALS AND METHODS

New Zealand white rabbits of 20 days' gestation weighing 3500 to 4500 g were obtained from a commercial source. The animals were divided into 2 groups and fed stock and experimental fat-cholesterol diets ad libitum as shown in table 1, with daily weighing of uneaten diet to determine the amount of diet eaten. The animals were fed the diets for 8 days beginning on day 20 of gestation. On day 28 of gestation they were anesthetized with pentobarbital (35 mg/kg) and the fetuses were delivered by opening the uterus. The fetal blood was obtained from the heart with siliconized needles attached to Tygon tubing; approximately 1 to 2 ml of blood were obtained from each fetus. All the fetal blood from the fetuses of each maternal rabbit was pooled to get enough fetal blood to carry out all analyses. Maternal blood was obtained

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[2] Presented in part at the Annual Meeting of the American Institute of Nutrition, Chicago, 1967.

TABLE 1

Composition of semipurified portion of fat-cholesterol and stock diet [1]

	Stock diet	Fat-cholesterol diet
	% by wt	*% by wt*
Peanut oil	2.0	20
(Total fat after mixing with pellets [2])	(2.5) [3]	(11.5) [3]
Choline chloride	0.5	0.5
Cholesterol	—	2.0
(Cholesterol after mixing with pellets [2])		(1.0) [3]
Casein	18.0	18.0
Salt mix [4]	6.0	6.0
Vitamin mix [5]	4.0	4.0
Sucrose	51.5	10.0
Cellulose [6]	18.0	39.5

Relative percentage of fatty acid composition of low fat stock diet and fat-cholesterol experimental diet after mixing with pellets

	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Stock diet	tr	16.6	2.2	3.0	34.0	35.0	7.9	tr
Fat-cholesterol diet	tr	13.2	0.48	3.0	45.3	35.3	1.8	tr

[1] The semipurified portions were mixed with an equal weight of Purina Rabbit Chow pellets (Lot # 5301, see footnote 2). On analysis for lipids in our laboratory the ration contained 3.08% total fat and no cholesterol, thus the final stock diet fed contained 2.5% fat and no cholesterol and the final fat-cholesterol diet fed contained 11.5% fat and 1% cholesterol. The caloric value of the 2 diets per gram was approximately the same.

[2] Purina Rabbit Chow Checkers, Ralston Purina Company, St. Louis. Contents stated by manufacturer on label: Guaranteed analysis: crude protein not less than 15.0%, crude fat not less than 2.0%, crude fiber not more than 18.0%. Ingredients: alfalfa meal, ground barley, ethoxyquin, wheat middlings, soybean meal, cane molasses, vitamin B_{12} supplement; D-activated animal sterol; vitamin E supplement; 0.2% calcium carbonate; 1.5% defluorinated phosphate; 0.1% mono-sodium phosphate; 0.25% iodized salt, and traces of iron oxide, manganese sulphate, manganous oxide, copper oxide, cobalt carbonate, zinc oxide.

[3] The figures in parentheses are the concentration of key ingredients in the final diet fed after mixing semipurified portion with pellets.

[4] Wesson, L. G. Science, 75: 339, 1932; obtained from Nutritional Biochemicals Corporation, Cleveland.

[5] Vitamin diet fortification mixture had the following composition per 100 g of semipurified portion of diet: (in grams) vitamin A conc (200,000 units/g), 0.018; vitamin D conc (400,000 units/g), 0.001; α-tocopherol, 0.020; ascorbic acid, 0.180; inositol, 0.020; menadione, 0.009; p-aminobenzoic acid, 0.020; niacin, 0.018; pyridoxine·HCl, 0.004; thiamine·HCl, 0.012; dextrose (triturated), 3.69; (in milligrams): biotin, 0.080; folic acid, 0.360; and vitamin B_{12}, 0.0054; obtained from Nutritional Biochemicals Corporation.

[6] Alphacel, nonnutritive bulk, otbained from Nutritional Biochemicals Corporation.

from the abdominal aorta with tubing and needles similar to those used to obtain fetal blood. Both maternal and fetal blood were mixed immediately with 1/9 volume of 1% sodium oxalate to prevent coagulation. The blood was immediately refrigerated and centrifuged at $900 \times g$ at 4° for 30 minutes to separate cells and plasma. The blood plasma samples were then placed in screw-cap vials with Teflon-lined caps and stored in liquid nitrogen until analyzed.

For analysis plasma samples were extracted by the method of Folch (4) using 2:1 chloroform-methanol at 20 times the volume of the plasma to be extracted. After one hour the samples were layered with 0.2 volume of 0.9% sodium chloride and allowed to stand overnight at 4°. Lipid phosphorus was determined by the method of Bartlett (5). Triglycerides were determined by the method of VanHandel and Zilversmit (6). Total cholesterol was determined by the Leffler (7) method. The cholesterol esters were determined by the Leffler method after digitonin precipitation and extraction with hexane by the Webster method (8). All chemical determinations were made in duplicate.

The fatty acid composition of the four major lipid fractions was determined by evaporating the chloroform-methanol extract of 1 to 2 ml of plasma under reduced pressure using a rotary evaporator, then taking it up in 1.0 ml of chloroform-methanol. This chloroform-methanol extract was applied with a Radin Pellick streaker [3] to a 20×20 cm glass plate coated with Adsorbosil 1 [4] 275-μ thick previously activated at 110° for 2 hours. These plates were developed by ascending chromatography using 80:20:2 hexane: ethyl ether:glacial acetic acid. Running time was about 40 minutes. The solvent was evaporated from the plates and they were sprayed with 2,7-dichlorofluorescein to mark the lipid bands. The lipid bands were scraped from the plate and placed in screw-cap vials with Teflon-lined caps. Methylation was carried out by adding 5.0 ml of methanol to each vial; then adding 0.4 ml of concentrated sulfuric acid. The vials were then refluxed at 65° for 2 hours with occasional agitation. After methylation, the methyl esters were extracted into hexane and analyzed on

[3] Obtained from Applied Science Laboratories, State College, Pennsylvania.

[4] See footnote 3.

a Barber-Colman Model 10 gas-phase chromatograph. The gas-phase chromatography machines were checked each morning with a standard methyl ester mixture.[5] Quantitative results agreed with stated composition data with a relative error of less than 4% for major components (greater than 7% of total mixture) and less than 7% for minor components (less than 7% of total mixture). Student's t test was used for the analysis of results with $P < 0.05$ being regarded as statistically significant.

RESULTS

The rabbits fed the stock diet ate an average of 65 g daily and the rabbits fed the fat-cholesterol diet, an average of 116 g daily. Thus the latter consumed about twice as many calories since the caloric content per gram of the 2 diets was approximately the same. The rabbits in the stock group gained an average of 24 g and those fed the fat-cholesterol diet, an average of 115 g.

The mean levels of plasma phospholipids, triglycerides, free and ester cholesterol of 11 maternal rabbits fed stock diet and 14 maternal rabbits fed fat-cholesterol and their fetuses are shown in table 2. The fetal plasma levels of all lipid classes in both stock and fat-cholesterol diet groups were higher than the maternal plasma levels. There were significant increases in the maternal plasma levels of phospholipids and free and ester cholesterol in the group fed fat-cholesterol when compared with the stock-fed group. The fetal plasma level of ester cholesterol was significantly increased in the group fed fat-cholesterol when compared with the stock-fed group.

Nonesterified fatty acids. Table 3 shows the mean relative percentage of fatty acid composition of the nonesterified fatty acid fraction of maternal and fetal plasma in the groups fed stock and fat-cholesterol diets. The only significant effect of the fat-cholesterol diet on the fatty acid composition of the nonesterified fatty acid fraction was an increase in the mean fetal plasma stearic acid from 6.9 in the stock group to 9.4 in the group fed fat-cholesterol.

Cholesterol esters. The mean relative percentage of fatty acid composition of the cholesterol esters of the plasma of maternal and fetal rabbits fed stock and fat-cholesterol diets is shown in table 4. The mean cholesterol palmitoleate level was significantly and approximately equally decreased in both maternal and fetal rabbit plasma of the group fed fat-cholesterol when compared with the levels in the stock-fed group. In the maternal plasma the mean palmitoleic decreased from 6.5 in the stock group to 3.9 in the fat-cholesterol group. In the fetal plasma it decreased from 10.6 in the stock group to 7.0 in the fat-cholesterol group. The mean relative percentage of cholesterol linoleate in the fetal plasma also decreased significantly to 20.9 in the group fed fat-cholesterol from 27.0 in the stock group.

[5] See footnote 3.

TABLE 2

Mean plasma lipid values of rabbits fed stock and fat-cholesterol diets and of their fetuses at 28 days' gestation

	No./group	Phospholipids	Triglycerides	Cholesterol	
				Ester	Free
		mg/100 ml	*mEq/liter*	*mg/100 ml*	*mg/100 ml*
Maternal					
Stock	11	1.1 ±0.4 [1]	2.0±0.7	8.9 ± 6.0	4.2 ± 2.6
Fat-cholesterol	14	2.2 [2]±0.7	1.9±0.7	38.7 [2]±21.2	10.9 [2]± 4.9
Fetal					
Stock	11	5.7 ±1.6	4.5±1.4	55.8 ±11.7	37.8 ±13.6
Fat-cholesterol	10	6.1 ±0.9	4.2±1.8	71.0 [3]± 8.1	39.5 ± 9.4

[1] Mean ± SD.
[2] Significantly different from stock value, $P < 0.001$.
[3] Significantly different from stock value, $P < 0.005$.

TABLE 3

Mean relative percentage of fatty acid composition of plasma nonesterified fatty acids of rabbits fed stock and fat-cholesterol diets and of their fetuses at 28 days' gestation

	No./group	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Maternal									
Stock	9	4.9 ± 1.7 [1]	37.4 ± 8.2	5.2 ± 2.2	7.1 ± 1.8	16.7 ± 3.8	10.8 ± 3.9	3.1 ± 1.3	2.9 ± 2.2
Fat-cholesterol	10	3.5 ± 2.0	31.4 ± 10.3	4.7 ± 1.4	7.1 ± 1.2	20.1 ± 7.1	12.9 ± 2.8	3.0 ± 1.8	1.5
Fetal									
Stock	11	4.8 ± 4.3	33.8 ± 5.8	6.5 ± 2.5	6.9 ± 1.7	14.2 ± 3.9	10.0 ± 3.8	2.8 ± 1.0	3.4 ± 1.8
Fat-cholesterol	9	7.6 ± 4.2	27.9 ± 9.5	6.1 ± 2.1	9.4 [2] ± 3.7	16.7 ± 5.2	8.2 ± 1.8	2.8 ± 0.6	4.0 ± 1.6

[1] Mean ± SD.
[2] Significantly different from stock value, $P < 0.05$.

TABLE 4

Mean relative percentage of fatty acid composition of plasma cholesterol esters of rabbits fed stock and fat-cholesterol diets and of their fetuses at 28 days' gestation

	No./group	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Maternal									
Stock	10	5.2 [1] ± 3.4 [2]	24.4 ± 6.1	6.5 ± 3.2	4.7 ± 1.8	14.2 ± 7.7	15.9 ± 10.6	2.5 ± 1.0	4.1 ± 1.3
Fat-cholesterol	9	2.8 [3] ± 2.6	21.7 ± 7.3	3.9 [4] ± 2.0	5.8 ± 1.4	39.2 [5] ± 14.2	17.7 ± 6.6	3.0 ± 2.2	2.5 ± 1.8
Fetal									
Stock	10	5.5 ± 1.2	24.0 ± 4.8	10.6 ± 3.5	2.0 ± 0.6	21.2 ± 2.7	27.0 ± 2.2	3.5 ± 4.5	4.5 ± 4.7
Fat-cholesterol	8	2.3 ± 1.9	31.0 [4] ± 9.5	7.0 [4] ± 1.6	1.8 ± 0.5	20.5 ± 3.7	20.9 [6] ± 6.5	1.5 ± 1.0	3.7 ± 1.5

[1] Only 9 calculable 14:0 peaks in the stock group.
[2] Mean ± SD.
[3] Only 8 calculable 14:0 peaks in the fat-cholesterol group.
[4] Significantly different from stock value, $P < 0.005$.
[5] Significantly different from stock value, $P < 0.001$.
[6] Significantly different from stock value, $P < 0.01$.

There was an increase in cholesterol palmitate in the fetal plasma of the fat-cholesterol group (31.0 vs. 24.0) compared with the stock-fed group and an increase in cholesterol oleate in the maternal plasma of the fat-cholesterol group (39.2 vs. 14.2) when compared with the stock-fed group.

Phospholipids. Table 5 shows the mean relative percentage of fatty acid composition of the plasma phospholipids in fetal and maternal rabbits fed stock and fat-cholesterol diets. In this fraction the mean relative percentage of arachidonic acid in the fat-cholesterol group fetal plasma was significantly lower (8.8) than the stock group fetal plasma (15.0).

Triglycerides. The mean relative percentage of fatty acid composition of the triglyceride fraction of material and fetal plasma lipids is shown in table 6.

In this lipid fraction there was a significant decrease in the fetal plasma triglyceride palmitoleate and an increase in the oleate and linoleate. The mean fetal plasma triglyceride palmitoleate decreased from 7.6 in the stock-fed group to 4.6 in the fat-cholesterol group. The mean fetal plasma triglyceride oleate increased from 24.4 in the stock group to 32.2 in the fat-cholesterol group. The mean fetal plasma triglyceride linoleate increased from 20.0 in the stock group to 24.2 in the fat-cholesterol group.

DISCUSSION

This study was carried out to investigate certain aspects of placental transport of lipids, as indicated by the extent to which maternal and fetal plasma lipid levels and their fatty acid composition could be affected by feeding pregnant rabbits diets differing in fatty acid composition and in amount of fat and cholesterol. An 8-day period of feeding the diet was felt to be adequate to develop maternal hypercholesterolemia, since it has been shown that in the rabbit the level of plasma lipids rises rapidly upon cholesterol administration. For example, in normal, nonpregnant rabbits an increase in total cholesterol from 72 mg/100 ml to 265 mg/100 ml in 48 hours after the administration of a single 1-g dose of cholesterol in water has been observed

TABLE 5

Mean relative percentage of fatty acid composition of plasma phospholipids of rabbits fed stock and fat-cholesterol diets and of their fetuses at 28 days' gestation

	No./group	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Maternal									
Stock	9	1.2 ± 1.1 [1]	28.6 ± 12.0	3.1 ± 3.9	21.7 ± 6.6	12.8 ± 8.5	17.0 ± 9.4	1.3 ± 0.7	6.5 ± 3.7
Fat-cholesterol	10	0.9 ± 0.6	29.1 ± 13.2	2.1 ± 0.3	19.2 ± 3.7	11.2 ± 2.9	22.9 ± 7.5	2.2 ± 1.4	8.4 ± 8.9
Fetal									
Stock	10	0.6 ± 0.5	23.5 ± 7.9	2.0 ± 0.8	19.5 ± 3.5	17.3 ± 4.2	21.3 ± 8.0	0.6 ± 0.6	15.0 ± 3.3
Fat-cholesterol	9	0.8 ± 0.6	26.4 ± 12.8	2.3 ± 1.6	16.6 ± 4.2	18.5 ± 3.5	19.0 ± 7.8	0.7 ± 0.2	8.8 [2] ± 3.9

[1] Mean ± SD.
[2] Significantly different from stock value, $P < 0.01$.

TABLE 6

Mean relative percentage of fatty acid composition of plasma triglycerides of rabbits fed stock and fat-cholesterol diets and of their fetuses at 28 days' gestation

	No./group	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Maternal									
Stock	10	3.2 ± 2.6 [1]	31.5 ± 10.7	4.3 ± 2.0	5.1 ± 2.0	25.1 ± 7.3	20.3 ± 7.9	2.6 ± 1.5	1.8 ± 0.9
Fat-cholesterol	10	1.7 ± 1.0	31.5 ± 9.0	3.0 ± 1.1	5.3 ± 1.8	29.6 ± 9.7	19.8 ± 0.3	1.8 ± 0.4	1.3 ± 0.4
Fetal									
Stock	10	3.2 ± 1.8	26.6 ± 5.3	7.6 ± 3.1	4.7 ± 2.0	24.4 ± 7.9	20.0 ± 4.8	3.0 ± 1.5	3.1 ± 1.6
Fat-cholesterol	9	2.9 ± 1.4	23.0 ± 8.9	4.6 [2] ± 2.2	6.0 ± 2.3	32.2 [2] ± 6.1	24.2 [2] ± 5.4	2.3 ± 1.1	3.1 ± 2.0

[1] Mean ± SD.
[2] Significantly different from stock value, $P < 0.05$.

(1). It has been shown that the levels of total cholesterol in nonpregnant rabbits can be raised from the normal range of 50 mg/100 ml to over 1000 mg/100 ml by feeding 1 g/day of cholesterol for 8 days (1, 2). In Popjak's study of pregnant rabbits he fed approximately 1 g/day of cholesterol from the eighth to the twenty-eighth day of pregnancy (3). By this method he was able to raise the total maternal plasma cholesterol from a mean of 15 mg/100 ml in his stock group to 109 mg/100 ml in his cholesterol-fed group. The mean total fetal plasma cholesterol in his study was raised from 110 mg/100 ml in the noncholesterol-fed to 144 mg/100 ml in his cholesterol-fed group.

The results of the portion of our study dealing with the effects of a fat-cholesterol diet on the levels of the plasma lipids in maternal and fetal rabbits confirm the observations of Popjak (3) that fetal lipid levels are not affected nearly as markedly as the maternal plasma lipids by feeding a fat-cholesterol diet to the maternal rabbit.

In addition to confirming the results of Popjak (3) and others (9), our study extends knowledge in this area of lipid metabolism and transport, provides new information on changes in levels and fatty acid composition of maternal and fetal plasma lipids and relates these changes to the amount of fat and fatty acid composition of the diets eaten.

The changes in the levels and fatty acid composition of the maternal plasma lipid fractions between the fat-cholesterol and stock diet groups correlate well with the amount of fat and the fatty acid composition of the 2 diets (table 1). The decreased maternal plasma cholesterol palmitoleate correlates with the decreased relative percentage of palmitoleate from 2.2 in the stock diet to 0.48 in the fat-cholesterol diet. This decrease in relative percentage of palmitoleate in the fat-cholesterol diet is due to the lack of palmitoleate in the peanut oil which supplied a larger amount of the fat in the fat-cholesterol diet than in the stock diet. The increased relative percentage of cholesterol oleate in the maternal plasma also correlates with the difference in the rel-

ative percentage of oleate in the 2 diets. The relative percentage of oleate in the fat-cholesterol diet is 45 as compared with 34 in the stock diet. The increases in maternal plasma levels of phospholipids, ester and free cholesterol in the group fed fat-cholesterol correlate with and are probably explained by the higher total amount of fat in this diet when compared with the stock diet.

In contrast with the good correlation of maternal plasma lipid levels and fatty acid composition with the amounts and fatty acid composition of the diet, there is poorer correlation of changes in fetal plasma lipid levels and fatty acid composition with dietary changes. The difference in response to dietary lipid alteration between maternal and fetal plasma lipids is likely due at least in part to the ability of the placenta to maintain the fetal plasma lipid levels and fatty composition at values relatively independent of those of the maternal plasma lipids.

The small but significant increase in fetal plasma cholesterol ester level in the fat-cholesterol group compared with the stock group correlates with the marked increase in maternal plasma cholesterol ester level. However, the increased fetal plasma cholesterol esters may not be causally related to the increased maternal plasma cholesterol esters, since the placenta is apparently only slightly permeable to cholesterol. In the human it has been shown that near term only about 1% of tritiated cholesterol crosses the placenta, whereas ^{14}C-acetate readily crosses the placenta and rapidly appears in the fetal plasma cholesterol (10). This is evidence that nearly all fetal cholesterol is formed de novo by the fetus from simple precursors and not obtained intact from the mother via the placenta.

In the rabbit it is possible that a small amount of preformed cholesterol does get across the placenta and that the amount of cholesterol crossing the placenta is sufficiently increased in the group fed fat-cholesterol to cause a rise in fetal plasma cholesterol ester levels. Another possible explanation for the increased fetal plasma cholesterol esters is an alteration in fetal metabolism of cholesterol esters resulting in a net increase in fetal plasma cholesterol ester levels.

Since the placenta has been shown to be nearly impermeable to phospholipids, it is not unexpected that there is no change in fetal plasma phospholipid levels despite changes in maternal plasma phospholipid levels. Popjak (11) found that ^{32}P-labeled phospholipids did not cross the placenta, but that inorganic ^{32}P was rapidly incorporated into fetal phospholipids. When ^{32}P-labeled phospholipids were given to the maternal rabbit there was very little radioactivity in the fetal phospholipids; however, there was greater specific activity in the placenta than when inorganic ^{32}P was given, indicating that the placenta apparently traps phospholipids and keeps them from crossing into the fetal blood.

In fetal plasma lipids there are eight significant changes in the relative percentage of fatty acid composition between the groups fed the stock and fat-cholesterol diets; three of these correlate directly with changes in the relative percentage of fatty acid composition of the 2 diets. Those that correlate with the dietary changes are the decreased cholesterol and triglyceride palmitoleate and the increased triglyceride oleate. The remainder of the changes in fetal plasma fatty acid composition do not appear to be associated directly with changes in dietary or maternal plasma fatty acid composition. These non-correlated changes are: increased nonesterified stearic acid, cholesterol palmitate, triglyceride linoleate, and decreased cholesterol linoleate and phospholipid arachidonate.

The mechanisms for the changes in fetal plasma lipid levels and plasma lipid fatty acid composition were not elucidated by this study. However, from our results and studies by others of placental transport and lipid metabolism during pregnancy some tentative conclusions can be drawn. There are two basic mechanisms by which the fetus can obtain its plasma lipids; the first is by transporting them intact across the placenta from the maternal plasma; the second is by synthesis of fetal plasma lipids de novo from simple precursors such as acetate and inorganic phosphorus. Both of these mechanisms

have been shown to play some role in providing fetal plasma lipids. As stated above, cholesterol is mostly synthesized de novo by the fetus; however, a small amount has been shown to be transported intact across the placenta (10).

There is some evidence that triglycerides cross the placenta in a manner similar to that noted in the gastrointestinal tract (12). This evidence is based on electron microscopic evidence of fat vacuoles in the syncytium after incubating placental tissue with sesame oil. However, these vacuoles have not been observed to pass through the basement membrane of the syncytium. Free fatty acids such as 1-^{14}C-palmitate (13) and 1-^{14}C-linoleate [6] have been shown to rapidly cross the placenta and are quickly incorporated into fetal lipids.

Apparently, the fetus derives most of its lipids by de novo synthesis from simple precursors obtained from the maternal plasma; however, to a small and possibly important extent some are transported as preformed lipids especially in the case of essential fatty acids such as linoleate. The level and relative percentages of fatty acids in the fetal plasma are probably regulated by a combination of 3 factors: 1) the rate of transport across the placenta; 2) the rate of synthesis of the lipid by the fetus; and 3) the rate of degradation or utilization of these lipids by the fetus. Without the use of radioactive tracer it is not possible to determine the contribution each of these mechanisms makes to the levels and fatty acid composition of the fetal plasma lipids. Decreased relative percentage of palmitoleate and the increased oleate in the triglyceride fraction of the fetal plasma indicate that triglycerides may be transported across the placenta because of the correlation of these with changes in dietary lipids. The changes in fatty acid composition in the fetal plasma lipids that are not correlated with dietary lipids or maternal plasma are more likely due to altered fetal synthesis or degradation, or both, than to altered placental transport.

Mechanisms accounting for these apparent changes in fetal lipid metabolism in response to maternal dietary changes are not clear. The maternal dietary alterations evidently produce changes in fetal metabolism through indirect multiple-step pathways in addition to changes that may be produced by direct transport of dietary material across the placenta.

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LITERATURE CITED

1. Popjak, B. 1946 The effects of feeding cholesterol without fat on the plasma lipids of the rabbit. The role of cholesterol in fat metabolism. Biochem. J., *40:* 608.
2. Kritchevsky, D., A. W. Mayer, W. C. Tesar, J. B. Logan, R. A. Brown, M. C. Davis and H. R. Cox 1954 Effect of cholesterol vehicle in experimental atherosclerosis. Amer. J. Physiol., *178:* 30.
3. Popjak, G. 1946 Maternal and foetal tissue and plasma lipids in normal and cholesterol-fed rabbits. J. Physiol., *105:* 236.
4. Folch, J., M. Lees and G. H. Sloane Stanley 1957 A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem., *226:* 497.
5. Bartlett, G. R. 1959 Phosphorus assay in column chromatography. J. Biol. Chem., *234:* 469.
6. VanHandel, E., and D. B. Zilversmit 1957 Micro method for direct determination of serum triglyceride. J. Clin. Med., *152:* 52.
7. Leffler, H. H. 1959 Estimation of cholesterol in serum. J. Clin. Pathol., *31:* 310.
8. Webster, D. 1962 The detemination of total and ester cholesterol in whole serum and plasma. Clin. Chim. Acta, *7:* 277.
9. Freidman, M., and S. O. Byers 1961 Effects of diets on serum lipids of fetal, neonatal and pregnant rabbits. Amer. J. Physiol., *201:* 611.
10. Davis, M. E., and E. J. Plotz 1957 The metabolism of progesterone and its clinical use in pregnancy. Recent Progr. Hormone Res., *13:* 347.
11. Popjak, G., and M. L. Beechmans 1950 Are phospholipids transmitted through the placenta? Biochem. J., *46:* 99.
12. Yamaguchi, R., Y. Okuda, K. Aneha and M. Mahano 1961 Experimental studies on the passage of fat through the human placenta. J. Jap. Obstet. Gynecol. Soc., *7:* 35.
13. Van Duyne, C. M., R. J. Havel and J. M. Felts 1962 Placental transfer of palmitic acid-1-C^{14} in rabbits. Amer. J. Obstet. Gynecol., *84:* 1069.

[6] Sisson, J. A., and E. J. Plotz, unpublished data, 1966.

Vitamin B_{12}, Choline and Related Substances in Dietary Hepatic Injury in Rats [1]

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ABSTRACT Choline and, to an even greater extent, methionine, were much less effective in preventing fatty infiltration of the liver in rats when given pair-fed as separate supplements than when mixed with the basal hypolipotropic diet; the basal diet contained a mixture of methanol-extracted peanut meal and casein, or peanut meal alone, as source of protein, with which young animals showed satisfactory initial gain in weight. These observations applied also to weight gain, food efficiency ratio and relative liver weight. The reduced beneficial effect of methionine given as separate supplement extends also to ceroid formation and fibrosis. The differences were less marked with vitamin B_{12} and became apparent only when vitamin B_{12} was given as a separate supplement to the ration containing the extracted peanut meal alone as source of protein. Vitamin B_{12} was comparable to choline in preventing fibrosis, but its lipotropic effect was weaker than that of choline or methionine (mixed with the diet). With the basal hypolipotropic ration containing casein and extracted peanut meal, vitamin B_{12} promoted growth, reduced the liver fat and prevented cirrhosis, even in the absence of folic acid. Anemia observed in rats fed the basal hypolipotropic diet was not prevented by homocystine, but was substantially improved by choline, methionine and vitamin B_{12} and to a somewhat less extent by cystine. Vitamin B_{12} stimulated growth even in animals receiving methionine, or homocystine and cystine in addition to choline. The relative liver weight was, in general, lowest in groups receiving vitamin B_{12}, in spite of higher fat content of the liver. The conclusion appears to be warranted that vitamin B_{12} acts not only by sparing lipotropic factors, or enhancing their synthesis, but also through other mechanisms.

In previous studies (1–3) the dietary factors effective in the prevention and treatment of experimental dietary cirrhosis in rats have been identified as lipotropic agents such as choline and its precursor, methionine. Proteins rich in methionine will act as lipotropic factors. Vitamin B_{12} has also been found to exert a lipotropic effect which, however, was weaker than that of choline or methionine (4–9). Vitamin B_{12} when combined with folic acid has a greater sparing effect on choline and its precursors (5, 6). As a lipotropic factor, vitamin B_{12} should also prevent dietary cirrhosis in rats. This assumption has been confirmed (4, 7). However, the effect of vitamin B_{12}, even when given together with folic acid as a supplement to a hypolipotropic diet low in protein, was found to be irregular (10, 11). The majority of the authors quoted assumed that vitamin B_{12} and folic acid participate in the synthesis of choline, reduce the requirement for choline and enhance the utilization of methionine and dimethylethanolamine. With a protein-free amino acid diet in which homocystine (or better homocysteine) replaced methionine (and cystine), addition of vitamin B_{12} in the presence of folic acid was found to promote growth, reduce fatty infiltration of the liver and partially prevent morphologic changes in the liver (cirrhosis, cholangiofibrosis). These injuries were accelerated and furthered by vitamin B_{12} deficiency (12, 13).

The experiments described above which dealt with lipotropism were of short duration. Those concerned with fibrosis and

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related pathologic changes of the liver required, by necessity, long-term observations. Dietary test supplements (choline, methionine, vitamin B_{12}, etc.) as well as all other vitamins were, with a few exceptions (1–4, 7, 12–14), mixed with the basal ration. Furthermore, the protein content of the rations (1–4, 7, 10, 11) or its equivalent in mixtures of amino acids (12, 13) was very low, not permitting satisfactory growth, partly due to the low intake of protein, partly to that of calories. More recent studies (15) have confirmed the original observation (16) that, with protein supplied as a mixture of methanol-extracted peanut meal (30%) and vitamin-test casein (6%), good growth could be achieved without interfering with the production of fatty liver and cirrhosis. With this diet or a slight modification of it (44% extracted peanut meal as sole source of protein) unexpected observations were made regarding the nutritional effect of supplements of choline, methionine, vitamin B_{12} and other related test supplements. The following report summarizes the findings and offers a tentative analysis of them.

EXPERIMENTAL METHODS

Hepatic injury was produced in young male rats of the Sprague-Dawley strain, with an average starting weight of 91 to 139 g in the various experiments. In a given experiment the average starting weight was identical in all groups. The basal ration contained either a combination of 6% vitamin-test casein and 25% methanol-extracted peanut meal in ration EPM V or methanol-extracted peanut meal alone (44%) in ration EPM VI as the source of protein. The protein content in the extracted peanut meal was 55%. Composition of the basal rations is given in table 1.

With EPM V an incomplete mixture of B vitamins (1–4) was given separately, 3 times weekly, in the calculated daily amount of thiamine·HCl, 20 μg; riboflavin, 25 μg; pyridoxine·HCl, 20 μg; Ca pantothenate, 100 μg; with added menadione, 20 μg. Ten milligrams of niacin were mixed with 100 g of the diet. In ration EPM VI, a complete mixture of B vitamins, except for choline and vitamin B_{12} which were to be used as test substances, was mixed with the diet.

TABLE 1

Composition of basal rations

	Diet EPM V	Diet EPM VI
	%	%
Extracted peanut meal	25	44
Casein (vitamin test)	6	—
Sucrose	45	31
Salt mixture, USP XIV [1]	4	4
Lard	18	18
Cod liver oil	2	—
Vitamins A, E, D in soya oil [2]	—	2
Vitamin mixture	— [3]	1 [4]

[1] Zinc acetate (67.5 mg/100 g diet) added to correct for its absence in the salt mixture.
[2] Vitamin A, 1250 IU; *dl-a*-tocopheryl acetate, 10 mg; vitamin D, 180 IU; dissolved in 2 ml soya oil.
[3] An incomplete mixture of vitamins was given separately, 3 times weekly providing per day: (in micrograms) thiamine·HCl, 20; riboflavin, 25; pyridoxine·HCl, 20; Ca pantothenate, 100; menadione, 20; dissolved in 1 ml water. Niacin was incorporated in the diet, 10 mg/100 g.
[4] Vitamin mixture contained: (in milligrams) thiamine·HCl, 0.5; riboflavin, 0.5; pyridoxine·HCl, 0.5; Ca pantothenate, 5; niacin, 8; p-aminobenzoic acid, 100; folic acid, 0.025; biotin, 0.02; ascorbic acid, 10; menadione, 0.5; inositol, 100; and sucrose to make 1 g.

In the first experiments to be discussed, the test substances were fed separately, with a daily dose of: vitamin B_{12}, 1 μg; choline chloride, 25 mg; methionine, 25 mg. In later experiments, supplements were mixed with the diet in such concentration as to give, per 100 g diet: vitamin B_{12}, 5 or 10 μg; choline chloride, 0.13 or 0.24 g; DL-methionine, 0.3 or 0.6 g; DL-homocystine, 0.69 or 1.38 g (equimolar with methionine); cystine, 0.49 g (equimolar with 0.3 g of methionine); and dimethylethanolamine, 0.085 g (equimolar with 0.13 g choline).

To avoid fatal cortical hemorrhagic necrosis of the kidneys, rats in the groups without supplements of choline, methionine or vitamin B_{12} received 10 mg of choline per day for the first 2 weeks of the experiment.

Feeding (with measured daily intake) was ad libitum except when specifically mentioned. Rats were kept in single cages, with raised wire bottoms. The duration of the experiments was, in general, 140 days. At the end of the experiment the rats were killed with ether and autopsied within one hour. Liver lipid was determined in chloroform-methanol extracts (17).

RESULTS

Methionine, when fed as a supplement to a hypolipotropic ration with a high level of protein (diet EPM V), failed to protect against hepatic fibrosis and ceroid formation (table 2). Liver fat was higher than in the control group. This is in contrast with the regular positive findings with methionine given in the same amount, but with a low casein diet as the basal ration (1). Supplements of crude liver extract and choline, however, resulted in complete protection against ceroid formation and fibrosis as in the past experiments; but choline in the same experiment has shown no prevention of fatty infiltration of the liver. Vitamin B_{12} given alone by injection was as effective as crude liver extract even with respect to weight gain. Rats receiving choline, and especially methionine as a separate supplement, showed significantly lower weight gain and food efficiency ratio (FER) than those given crude liver extract or vitamin B_{12}, although higher than the control group.

Table 3 summarizes the results of a test of vitamin B_{12} in a therapeutic experiment. Two groups of rats were fed the basal ration (EPM V) without supplement for 12 weeks. After this initial period, one group received vitamin B_{12}, 1 μg per day; the other group remained as control. After 20 additional weeks only 3 rats in the treated group had more than traces of ceroid and fibrosis, whereas all rats in the control group had severe fibrosis with ceroid. The FER, calculated for the last 20 weeks, was significantly higher in the treated than in the control group.

The dose response relation to vitamin B_{12} varied with the parameter studied (table 4). A plateau was reached for weight gain and FER with 0.1 μg of vitamin B_{12}. The same amount protected completely against hepatic fibrosis and formation of ceroid. Less (0.03 μg) was needed to bring the size of the liver (as percentage of total body weight) to the minimal value, whereas only with 0.3 μg was liver fat (expressed as percentage of fresh liver) at a normal value.

In an experiment in which the effects of supplements given separately and mixed with the diet (EPM V) were compared (table 5), the earlier observation (table 2) that methionine, as a separate supplement, was ineffective in the prevention of hepatic injury, was confirmed; mixed with the diet however, it prevented fatty liver, fibrosis and formation of cer-

TABLE 2

Effect of lipotropic supplements on weight gain, food efficiency ratio and liver of rats fed diet EPM V

Group	Supplement [1]	Wt gain [2]	FER [3]	Liver				
				Total lipid	Ceroid		Fibrosis	
					No. of rats		No. of rats	
					Without	With	Without	With
		g	*g gain/100 g food*	*%*				
1	None	107 ± 12.6 [4]	8.0 ± 0.6 [5]	19.1 ± 2.45	0	10	0	10
2	Vitamin B_{12}, 1.0 μg/day	311 ± 9.5	16.8 ± 0.2	8.0 ± 0.96	10	0	10	0
3	Crude liver extract,[6] 20 mg/day	310 ± 7.7	16.0 ± 0.3	—	10	0	10	0
4	Choline chloride, 25 mg/day	228 ± 10.9	12.7 ± 0.4 [5]	26.5 ± 0.98	10	0	10	0
5	Methionine, 25 mg/day	175 ± 12.7	10.6 ± 0.6 [5]	28.0 ± 0.95	0	10	3	7

[1] Supplements given 3 times/week orally, except for vitamin B_{12} which was injected daily.
[2] Average starting weight in each group, 91 g; duration of experiment, 140 days.
[3] FER indicates food efficiency ratio: gain in weight/100 g of food.
[4] Values are average ± SE.
[5] Highly significant difference from group 2 ($P < 0.01$).
[6] BL, Wilson Company, Chicago; vitamin B_{12} content: 0.14 μg/20 mg BL.

TABLE 3

Therapeutic effect of vitamin B_{12} in rats fed hypolipotropic diet (EPM V)

Group	Supplement	Initial wt	Wt gain	FER	Liver			
					Ceroid		Fibrosis	
					No. of rats		No. of rats	
					Without	With	Without	With
		g	*g*	*g gain/100 g of food*				
		First 12 weeks of experiment						
1	None	136±2.7 [1]	156±7.6	15.5±0.8				
2	None	136±1.7	163±9.4	15.6±0.8				
		Succeeding 20 weeks of experiment						
1	Vitamin B_{12} [2]	292±8.1	120±7.0 [3]	5.7±0.4 [3]	7	3	7	3
2	None	299±9.2	35±7.0	1.8±0.3	0	10	0	10

[1] Average ± SE.
[2] 1 μg/day, injected.
[3] Highly significant difference from group 2 ($P < 0.01$).

oid. Methionine mixed with the diet also gave significantly higher weight gain and FER. Choline, mixed with the diet or given separately, caused reduction in liver fat concentration; however, the effect was significantly less in the group receiving choline separately. The effect of vitamin B_{12} was the same whether it was given mixed with the diet, by injection, or separately by mouth. The only exception was the significantly higher FER with vitamin B_{12} mixed with the diet compared with the other 2 groups. In this (table 5) and in the following experiment (table 6) the groups fed the supplements separately were pair-fed, with respect to the supplements, with the groups receiving the supplements mixed with the diet.

The difference in the effectiveness of supplements given separately and mixed with the diet was more pronounced when diet EPM VI replaced diet EPM V as basal ration (table 6). This diet contained no casein but only extracted peanut meal as the protein source, and a complete vitamin mixture instead of the restricted mixture of diet EPM V. With methionine given separately, all parameters showed greatly reduced effect. Even when it was mixed with the diet the relative liver weight and the liver fat concentration were significantly higher than observed with choline mixed with the diet, in contrast with the result with diet EPM V. Choline given separately was equivalent to choline in the diet only in prevention of ceroid and fibrosis. With vitamin B_{12}, differences were noted in liver weight, liver fat concentration and hepatic fibrosis, all showing reduced effect with separate administration of the supplement; there were no differences in weight gain or FER.

Later experiments (tables 7 and 8) included, in addition to the supplements used previously, cystine, homocystine and dimethylethanolamine, tested in various combinations. The basal diet was EPM VI and all supplements were mixed with the diet. The salient results may be summarized as follows. a) Cystine and homocystine had no preventive effect on hepatic injury (fatty infiltration, ceroid formation and fibrosis). b) Dimethylethanolamine reduced slightly but significantly liver fat and prevented completely ceroid formation and fibrosis. c) Vitamin B_{12} protected the liver against the formation of ceroid and fibrosis. Its lipotropic effect, although significant, was not as marked as that of choline and methionine. However, vitamin B_{12} synergistically increased the lipotropic effect of dimethylethanolamine and also that of choline given in combination with homocystine or cystine. Vitamin B_{12} had a significant effect on weight gain, especially when it was given in combination with cystine or homocystine and choline there was a marked stimulation of growth above that produced by those supplements without vitamin B_{12}. With vitamin B_{12} the fresh liver weight expressed as percentage

TABLE 4

Dose response relation to supplements of vitamin B_{12} [1]

Group	Vitamin B_{12} injected	Wt gain [2]	FER	Liver					
				Weight	Total lipid	Ceroid		Fibrosis	
						No. of rats		No. of rats	
						Without	With	Without	With
	µg/day	*g*	*g gain/100 g of food*	*% of body wt*					
1	None	147 ± 9.6 [3,4]	9.4 ± 0.5	10.6 ± 0.4	27.8 ± 1.0	0	10	0	10
2	0.03	225 ± 3.9	12.8 ± 0.2 [6]	5.2 ± 0.3 [6]	17.0 ± 1.1 [5]	5	5	6	4
3	0.1	259 ± 6.2 [4]	14.1 ± 0.3 [6]	4.7 ± 0.2 [6]	12.0 ± 0.8	10	0	10	0
4	0.3	263 ± 7.2 [4]	13.9 ± 0.3 [6]	4.3 ± 0.1 [6]	9.3 ± 1.2 [5]	10	0	10	0
5	1.0	256 ± 6.3 [4]	13.2 ± 0.2 [6]	4.4 ± 0.1 [6]	7.1 ± 0.4 [5]	10	0	10	0

[1] Diet EPM V; duration of experiment, 140 days.
[2] Average initial weight in each group, 107 g.
[3] Values are average ± SE.
[4] Highly significant difference from group 2 ($P < 0.01$).
[5] Highly significant difference from group 3 ($P << 0.01$).
[6] Highly significant difference from group 1 ($P << 0.01$).

of body weight was in the range of the lowest values observed in any group, in spite of the significantly higher liver fat concentration. d) Cystine did not cause a significant increase in weight compared with the control groups. Homocystine and dimethylethanolamine were effective in stimulating weight gain, but less so than methionine, choline and vitamin B_{12}. e) The lowest hematocrit values were found in the control groups and in those receiving homocystine. In all other groups the values were close to or in the normal range, with some reduction in the group receiving cystine.

One experiment was set up with the purpose maintaining the groups of rats one year with a basal ration (EPM VI), a) without supplement; b) with 1 µg vitamin B_{12} injected; c) 25 mg choline·Cl given separately; or d) 25 µg folic acid given separately. The supplements were given daily. All rats in groups *a* and *d* died within 45 weeks. Only 2 rats survived in group c (with choline), whereas all rats in group *b* (with vitamin B_{12}) survived 52 weeks. One rat in the choline group died early from pneumonia, five of the remaining rats in this group showed fibrosis. All rats in the control group and in the group with folic acid exhibited fibrosis; none in the 10 surviving rats with vitamin B_{12}.

COMMENTS

Several observations reported in these experiments appear to require some modifications of previous concepts on the nutritive effect of lipotropic factors and similar compounds in growing rats.

The prevention of liver injury by choline, methionine and vitamin B_{12} was first demonstrated (1–3) in rats receiving a hypolipotropic diet low in protein (8–10% casein). The supplements of choline, methionine and vitamin B_{12} (or crude liver extract) were fed separately in those experiments. The effect extended not only to the reduction of liver fat but also to the prevention of hepatic fibrosis (cirrhosis). In the present studies, with the use of different basal hypolipotropic rations which were high in protein (methanol-extracted peanut meal, with or without casein), the beneficial effect of choline and particularly that of methionine became in several as-

TABLE 5

Variation in response of rats fed a hypolipotropic diet [1] to lipotropic supplements fed separately or incorporated into diet

Group	Supplement [2]	Wt gain [3]	FER	Liver					
				Weight	Total lipid	Ceroid		Fibrosis	
						No. of rats		No. of rats	
						Without	With	Without	With
		g	*g gain/100 g of food*	*% of body wt*	*%*				
1	None	101 ± 10.5 [4]	6.5 ± 0.5	12.0 ± 0.5	18.2 ± 1.4	0	10	0	10
2	Methionine, in diet	248 ± 6.3	13.3 ± 0.2	5.2 ± 0.3	4.6 ± 0.2	10	0	10	0
3	Methionine, fed separately	191 ± 9.0 [5]	10.0 ± 1.1 [5]	9.3 ± 0.4 [5]	25.0 ± 0.8 [5]	1	9	4	6
4	Choline, in diet	227 ± 7.4	12.7 ± 0.3	4.9 ± 0.2	4.1 ± 0.2	10	0	10	0
5	Choline, fed separately	237 ± 7.9	11.3 ± 0.5	5.2 ± 0.3	5.9 ± 0.4 [5]	10	0	10	0
6	Vitamin B_{12}, in diet	248 ± 5.8	13.2 ± 0.3	5.0 ± 0.1	6.1 ± 0.8	8	2 [6]	10	0
7	Vitamin B_{12}, fed separately	252 ± 6.8	12.0 ± 0.2 [5]	4.9 ± 0.1	6.1 ± 0.6	4	6 [6]	10	0
8	Vitamin B_{12}, injected	241 ± 5.3	11.8 ± 0.3	4.6 ± 0.1	5.5 ± 0.6	8	2 [6]	10	0

[1] Diet EPM V (incomplete B vitamin mixture).

[2] Concentration of supplements in the diet: methionine, 0.3%; choline chloride, 0.24%; vitamin B_{12}, 5 μg/100 g. Separate oral supplements were given 3 times weekly. Vitamin B_{12} was injected daily. Groups 2 and 3, 4 and 5, and 6, 7 and 8 were pair-fed with respect to supplement.

[3] Average initial weight, 91 g in each group; duration of experiment, 140 days.

[4] Values are mean ± SE.

[5] Highly significant difference from value with same supplement incorporated in the diet.

[6] Traces only.

TABLE 6

Variation in response of rats fed a hypolipotropic diet [1] to lipotropic supplements fed separately or incorporated into the diet

Group	Supplement [2]	Wt gain [3]	FER	Liver					
				Weight	Total lipid	Ceroid		Fibrosis	
						No. of rats		No. of rats	
						Without	With	Without	With
		g	*g gain/100 g of food*	*% of body wt*	*%*				
1	None	109 ± 10.8 [4]	6.4 ± 0.5	7.4 ± 0.2	18.7 ± 0.6	0	9	0	9
2	Choline, in diet	282 ± 7.4	12.0 ± 0.2	5.1 ± 0.1	3.9 ± 0.2	10	0	10	0
3	Choline, fed separately	233 ± 3.7 [5]	10.2 ± 0.3 [5]	5.9 ± 0.2 [5]	9.0 ± 0.8 [5]	10	0	10	0
4	Methionine, in diet	270 ± 6.2	11.5 ± 0.1	6.6 ± 0.3	14.4 ± 1.8	10	0	10	0
5	Methionine, fed separately	153 ± 8.5 [5]	8.7 ± 0.4 [5]	7.9 ± 0.1 [5]	23.1 ± 1.1 [5]	0	10	1	9
6	Vitamin B_{12}, in diet	351 ± 11.4	14.1 ± 0.4	3.9 ± 0.1	6.9 ± 0.4	9	1	9	1
7	Vitamin B_{12}, fed separately	340 ± 11.0	13.8 ± 0.4	4.4 ± 0.2 [6]	9.7 ± 0.8 [5]	10	0	1	9 [7]

[1] Diet EPM VI (complete B vitamin mixture).

[2] Concentration of supplements in the diet: methionine, 0.3%; choline chloride, 0.24%; vitamin B_{12}, 5 μg/100 g. Separate oral supplements were given 3 times weekly. Groups 2 and 3, 4 and 5, and 6 and 7 were pair-fed with respect to supplement.

[3] Average initial weight for each group, 107 g; duration of experiment, 140 days.

[4] Values are average ± SE.

[5] Highly significant difference from value with same supplement incorporated in the diet ($P < 0.01$).

[6] Significant difference from value with same supplement incorporated in the diet ($P < 0.05$).

[7] Mostly in traces.

TABLE 7

Effect of homocystine and dimethylethanolamine, vitamin B_{12} and methionine alone or in combination, as supplements to diet EPM VI

Group	Supplements [1]	Wt gain [2]	FER	Liver					
				Weight	Total lipid	Ceroid No. of rats		Fibrosis No. of rats	
						Without	With	Without	With
		g	*g gain/100 g of food*	*% of body wt*	%				
1	None	135 ± 7.2 [3,4]	8.8 ± 0.4 [4]	8.2 ± 0.3 [4]	27.1 ± 0.5 [4]	0	10	0	10
2	Vitamin B_{12}	381 ± 11.4	16.3 ± 0.4	3.6 ± 0.1	8.0 ± 0.5	9	0	9	0
3	M	314 ± 8.0 [4]	14.4 ± 0.4 [4]	3.9 ± 0.1	6.2 ± 0.2 [4]	9	0	9	0
4	M + vitamin B_{12}	379 ± 8.0	16.0 ± 0.3	3.4 ± 0.1	6.5 ± 0.2 [5]	10	0	10	0
5	H	216 ± 6.4 [4]	11.0 ± 0.2 [4]	9.5 ± 0.4 [4]	25.0 ± 0.8 [4]	1	9	1	9
6	H + vitamin B_{12}	423 ± 10.8 [5]	18.0 ± 0.6 [5]	4.1 ± 0.1 [5]	8.2 ± 0.6	10	0	10	0
7	C	341 ± 9.9 [5]	15.4 ± 0.4	4.1 ± 0.1 [5]	6.0 ± 0.3 [4]	8	0	8	0
8	C + vitamin B_{12}	384 ± 6.2	16.0 ± 0.3	3.6 ± 0.1	6.1 ± 0.2 [4]	10	0	10	0
9	D	230 ± 5.7 [4]	11.9 ± 0.2 [4]	6.4 ± 0.1 [4]	15.3 ± 0.6 [4]	8	0	8	0
10	D + vitamin B_{12}	392 ± 9.2	17.1 ± 0.1	3.4 ± 0.1	5.9 ± 0.2 [4]	10	0	10	0
11	D + M	339 ± 10.4 [5]	16.1 ± 0.4	3.9 ± 0.2	6.1 ± 0.2 [4]	10	0	10	0
12	D + M + vitamin B_{12}	383 ± 10.4	16.0 ± 0.3	3.7 ± 0.1	6.7 ± 0.2	10	0	10	0
13	D + H	306 ± 17.0 [4]	13.9 ± 0.5 [4]	6.8 ± 0.3 [4]	18.6 ± 0.8 [4]	9	0	9	0
14	D + H + vitamin B_{12}	403 ± 12.6	17.2 ± 0.2	3.9 ± 0.1	5.8 ± 0.2 [4]	9	0	9	0
15	C + H	340 ± 9.6 [5]	15.8 ± 0.4	4.6 ± 0.2 [4]	6.3 ± 0.2 [4]	10	0	10	0
16	C + H + vitamin B_{12}	423 ± 8.0 [4]	17.0 ± 0.3	3.9 ± 0.1	5.8 ± 0.2 [4]	10	0	10	0
17	C + M	362 ± 13.6	15.4 ± 0.4	3.9 ± 0.1	5.9 ± 0.2 [4]	10	0	10	0
18	C + M + vitamin B_{12}	386 ± 12.2	16.5 ± 0.3	3.3 ± 0.2	5.7 ± 0.2 [4]	8	0	8	0

[1] All supplements added to the diet. Per 100 g of diet: vitamin B_{12}, 10 μg; choline chloride (C), 0.13 g; DL-methionine (M), 0.6 g; dimethylethanolamine (D), 0.85 g; DL-homocystine (H), 0.69 g.

[2] Average initial weight, 105 g in each group; duration of experiment, 140 days.

[3] Values are average ± SE.

[4] Highly significant difference from group 2 ($P < 0.01$).

[5] Significant difference from group 2 ($P < 0.05$).

TABLE 8

Effect of homocystine, cystine, choline, methionine and vitamin B_{12} alone, and in combination, as supplements to diet EPM VI

Group	Supplements [1]	Wt gain [2]	FER	Liver						Hematocrit
				Weight	Total lipid	Ceroid No. of rats		Fibrosis No. of rats		
						Without	With	Without	With	
		g	*g gain/100 g of food*	*% of body wt*	%					%
1	None	100 ± 7.6 [3,4]	6.2 ± 0.3	6.7 ± 0.2 [4]	20.4 ± 1.0 [4]	1	9	0	10	33.9 ± 1.2 [4]
2	Vitamin B_{12}	301 ± 10.5	14.1 ± 0.5	4.0 ± 0.3	9.4 ± 1.3	9	1	9	1	53.0 ± 2.0
3	C	323 ± 9.9	14.6 ± 0.3	3.6 ± 0.1	5.7 ± 0.4 [5]	10	0	10	0	55.6 ± 1.0
4	C + vitamin B_{12}	327 ± 17.7	14.8 ± 0.3	3.3 ± 0.1 [5]	5.0 ± 0.2 [4]	10	0	10	0	57.0 ± 1.8
5	Cy	139 ± 13.9 [4]	7.3 ± 0.4	7.4 ± 0.2 [4]	20.5 ± 0.9 [4]	1	9	1	9	50.8 ± 1.5
6	Cy + C	275 ± 11.8	12.9 ± 0.3	4.7 ± 0.1 [5]	7.0 ± 0.5	10	0	10	0	61.4 ± 1.7 [4]
7	Cy + C + vitamin B_{12}	347 ± 12.1 [5]	15.6 ± 0.3	3.4 ± 0.1	4.9 ± 0.2 [4]	10	0	10	0	55.1 ± 0.7
8	Cy + vitamin B_{12}	345 ± 9.8 [4]	15.3 ± 0.3	4.0 ± 0.1	8.6 ± 0.7	10	0	10	0	56.5 ± 1.2
9	H	151 ± 8.6 [4]	8.4 ± 0.4	7.8 ± 0.1 [4]	19.5 ± 0.6 [4]	0	10	0	10	43.7 ± 2.2 [4]
10	H + C	255 ± 7.8 [4]	12.6 ± 0.2	4.6 ± 0.1	5.2 ± 0.3 [4]	10	0	10	0	53.8 ± 1.3
11	H + C + vitamin B_{12}	323 ± 23.4	14.6 ± 0.8	3.9 ± 0.1	4.0 ± 0.2 [4]	9	0	9	0	55.4 ± 1.1
12	H + vitamin B_{12}	328 ± 15.2	14.7 ± 0.5	4.3 ± 0.1	7.1 ± 0.7	10	0	10	0	53.2 ± 1.1
13	M + C	335 ± 11.9 [5]	14.8 ± 0.3	3.5 ± 0.1	4.9 ± 0.2 [4]	10	0	10	0	52.6 ± 0.7
14	M + C + vitamin B_{12}	334 ± 10.3 [5]	15.1 ± 0.4	3.4 ± 0.1	4.9 ± 0.2 [4]	10	0	10	0	53.8 ± 0.6

[1] All supplements incorporated in the diet; per 100 g of diet: vitamin B_{12}, 10 μg; choline chloride (C), 0.13 g; DL-methionine (M), 0.6 g; DL-homocystine (H), 1.08 g; cystine (Cy), 0.49 g.

[2] Average initial starting weight in each group, 139 g; duration of experiment, 140 days.

[3] Values are average ± SE.

[4] Highly significant difference from group 2 ($P < 0.01$).

[5] Significant difference from group 2 ($P < 0.05$).

pects less pronounced — for methionine often was completely absent — when the supplements were administered separately.

A difference in the effect of choline mixed with the diet in the amount of 0.24% and the same amount fed separately was evident and significant but not striking in its lipotropic effect when ration EPM V was used (table 5). The difference was very marked in weight gain, food efficiency ratio, relative liver weight and liver fat with EPM VI (table 6). A separate dose of 25 mg per day gave only incomplete protection: cirrhosis did not develop but liver fat was very high (table 2). A level of 0.13% of choline in the diet gave complete protection although at this concentration the daily choline intake never reached 25 mg (18, 20, 21, 21, 23 mg) in the five 4-week periods of the experiment (table 7).

With methionine, 0.3% even when mixed with the diet (EPM VI), containing 22% protein all supplied by extracted peanut meal, fibrosis was prevented but not fatty liver (table 6). The supplement of methionine raised the concentration of this limiting factor in peanut protein to 0.52% (18), higher than the amount recommended in the FAO amino acid pattern, 0.48%. Complete protection with the same basal ration (EPM VI) was achieved only with a concentration of 0.6% (tables 7 and 8), a level which exceeds the FAO pattern by a large margin. It may be assumed that part of the methionine in the diet (19), a larger proportion of it than when fed as a separate supplement (20, 21), will be used for protein formation in support of growth. With 0.3% methionine mixed with the diet EPM VI, not enough would be left over for methylation processes and thus for sufficient lipotropic effect. The fact that 0.3% methionine, mixed with the diet EPM, which contains 6% casein and thus a higher methionine level, was in every respect effective in preventing hepatic injury (table 6), is in good accord with these considerations. But how then may one explain the almost complete absence of any specific effect, with only slight increase in weight, when methionine was given separately? In this case methionine as source of labile methyl groups should have been more active than when mixed with the diet. The same question applies also to choline. One obvious possibility is that choline and methionine, given separately, are destroyed more rapidly by intestinal bacteria (22, 23). A practical, clinical application suggested by these experiments would be the preference of mixing supplements like choline, methionine and vitamin B_{12} with the diet instead of giving them, as is customary in medical practice, as separate supplements. The negative clinical results with lipotropic substances in liver diseases may also have a close relation to the experimental observations here presented.

Cystine and homocystine exerted no lipotropic effect; dimethylethanolamine (alone or with homocystine) slightly only. However, dimethylethanolamine, but not cystine or homocystine, prevented formation of ceroid and fibrosis. Homocystine, but not cystine promoted growth significantly and produced a better food efficiency ratio. The previously expressed view that cystine aggravates choline deficiency due to higher food intake and better growth (25) is not confirmed by these observations (table 8). In contrast with homocystine, cystine supports normal hematocrit values.

Vitamin B_{12} exerts demonstrable lipotropic effect and promotes growth even in the absence of folic acid (tables 2–4); this is in contrast with previous findings (5, 6) with the same diet used in these studies. The general view that vitamin B_{12} enhances the synthesis of choline (methyl transfer) is only partially borne out by the present studies. Mixing vitamin B_{12} with the diet had no advantage over feeding it separately when rations containing animal protein such as casein were used (2–4; table 5); with extracted peanut meal as the sole source of protein, vitamin B_{12} had more effect when it was incorporated in the diet than when it was given as a separate supplement (table 6). In contrast with previous observations (10, 11) and in spite of incomplete lipotropic effect, vitamin B_{12} was found regularly beneficial for the prevention of fibrosis even after 52 weeks in the experiment. The growth-promoting effect of vitamin B_{12} was impressive, not only when it was given as the only supplement, but when

it was used as an additional supplement with choline, methionine, cystine, homocystine, dimethylethanolamine and combinations of these substances. The same was generally true of the FER. With supplements of vitamin B_{12}, the average liver weight (as percentage of body weight) had a very low value, often the lowest in the whole series, while liver fat did not reach the low values observed with choline and methionine mixed with the diet.

The results of the therapeutic experiment (table 3) have to be related to our previous observations on obvious regression of already existing dietary cirrhosis under treatment with lipotropic factors, especially crude liver extract (3). The high incidence of death of rats in which for diagnostic reasons pre-treatment liver biopsy was done (3), deterred us from this surgical procedure in the present study.

A need for revision of older views is supported by these observations. The outstanding influence of vitamin B_{12} on weight gain and on reduced relative liver weight (this probably related to the overriding stimulation of weight gain) without maximal lipotropic effect indicates that the role of vitamin B_{12} under the experimental conditions reported is not fully explained by stimulation of choline synthesis or methyl transfer but must have an additional explanation such as synthesis of nucleotides or proteins. In conclusion, vitamin B_{12} appears to emerge as the central factor in the underlying metabolic reactions and therefore, also in the etiology of dietary liver injury.

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LITERATURE CITED

1. György, P., and H. Goldblatt 1942 Observations on the conditions of dietary hepatic injury (necrosis, cirrhosis) in rats. J. Exp. Med., *75:* 355.
2. György, P., and H. Goldblatt 1949 Further observations on the production and prevention of dietary hepatic injury in rats. J. Exp. Med., *89:* 245.
3. György, P., and H. Goldblatt 1949 Treatment of experimental dietary cirrhosis of the liver in rats. J. Exp. Med., *90:* 73.
4. György, P., and C. S. Rose 1950 Effect of vitamin B_{12} on experimental hepatic injury. Proc. Soc. Exp. Biol. Med., *73:* 372.
5. Schaefer, A. E., and J. L. Knowles 1951 Influence of vitamin B_{12} and folacin on the synthesis of choline and methionine by the rat. Proc. Soc. Exp. Biol. Med., *77:* 655.
6. Strength, D. R., A. E. Schaefer and W. D. Salmon 1951 The relation of vitamin B_{12} and folacin to the utilization of choline and its precursors. J. Nutr., *45:* 329.
7. György, P. 1951 Effects of antibiotics and vitamin B_{12} in cirrhosis and necrosis of the liver. In: Liver Diseases, Ciba Foundation Symposium. The Blakiston Company, Philadelphia, p. 81.
8. Best, C. H., C. C. Lucas, J. M. Patterson and J. H. Ridout 1953 Some effects of vitamin B_{12} in weanling rats consuming hypolipotropic diets. Can. J. Med. Sci., *31:* 135.
9. Hawk, E. A., and C. A. Elvehjem 1953 The effects of vitamin B_{12} and B_{12f} on growth, kidney hemorrhage and liver fat in rats fed purified diets. J. Nutr., *49:* 495.
10. Alexander, H. D., and R. W. Engel 1952 The importance of choline in the prevention of nutritional edema in rats fed low-protein diets. J. Nutr., *47:* 361.
11. Alexander, H. D., and H. E. Sauberlich 1957 The influence of lipotropic factors on the prevention of nutritional edema in the rat. J. Nutr., *61:* 329.
12. Bennett, M. A., J. Joralemon and P. E. Halpern 1951 The effect of vitamin B_{12} on rat growth and fat infiltration of the liver. J. Biol. Chem., *193:* 285.
13. Bennett, M. A., J. Hellerman and A. J. Donnelly 1955 Influence of vitamin B_{12} on liver disease due to a "labile methyl" free diet. Cancer Res., *15:* 398.
14. Engel, R. W., and W. D. Salmon 1941 Improved diets for nutritional and pathologic studies of choline deficiency in young rats. J. Nutr., *22:* 109.
15. György, P., H. Goldblatt and M. Ganzin 1959 Dietary fat and cirrhosis of the liver. Gastroenterology, *37:* 637.
16. Copeland, D. H., and W. D. Salmon 1946 Occurrence of neoplasms in the liver, lungs and other tissues of rats as a result of prolonged choline deficiency. Amer. J. Pathol., *22:* 1059.
17. Bragdon, J. H. 1960 In: Lipids and Steroid Hormones in Clinical Medicine, eds., F. W. Sunderman and F. W. Sunderman, Jr. J. B. Lippincott Company, Philadelphia, p. 11.
18. Milner, M. 1962 Peanuts as a protein source in international feeding programs. Food Technol., *16:* 46.
19. Young, R. J., C. C. Lucas, J. M. Patterson and C. H. Best 1956 Lipotropic dose response studies in rats: comparison of choline, betaine and methionine. Can. J. Biochem. Physiol., *34:* 713.
20. Cuthbertson, D. P., A. McCutcheon and H. N. Munro 1940 The relationship of carbohydrate metabolism to protein metabo-

lism. II. Note on the effect of separation of time of feeding the protein and carbohydrate moieties of the adult and growing rats. Biochem. J., 34: 1002.
21. Geiger, E., 1948 The importance of the time element in feeding of growing rats. Experiments with delayed administration of protein. Science, *108*: 42.
22. De la Huerga, J., P. György, S. Waldstein, R. Katz and H. Popper 1953 Effects of antimicrobial agents upon choline degradation in the intestinal tract. J. Clin. Invest., 32: 1117.
23. Phear, E. A., B. Ruebner, S. Sherlock and W. H. Summerskill 1956 Methionine toxicity in liver disease and its prevention by chlortetracycline. Clin. Sci., *15*: 93.
24. Mulford, D. J., and W. H. Griffith 1942 Choline metabolism. VIII. The relation of cystine and methionine to requirement of choline in young rats. J. Nutr. 23: 91.

Assimilation by Rats of Limiting Amino Acid into Protein from Imbalanced Dietary Sources [1]

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ABSTRACT The hypothesis that elevated efficiency in converting the limiting amino acid into tissue protein is the cause of the depressed concentrations of free amino acids in the circulation after ingestion of an imbalanced mixture of amino acids was tested. Diets imbalanced and corrected with respect to histidine were prepared using histidine-^{15}N. Over the first 6 hours following feeding, the ^{15}N in the protein and the free amino acid pools of various tissues were determined. Observation of enhanced efficiency of assimilation of dietary histidine into protein affirmed the validity of the hypothesis.

An hypothesis to explain the amino acid imbalance phenomenon was formalized by Harper and his group (1) as follows: "... the amino acid mixture added to create an imbalance in some way depresses the plasma concentration of the limiting amino acid, causing the blood amino acid pattern to resemble that of an animal fed a much more deficient diet. This then, in some way, triggers an appetite-depressing mechanism, and food intake falls."

The hypothesis was a significant contribution because it appeared to be compatible with all existing data on amino acid imbalance of quite varied nature and because it provoked direct experimental testing.

Just how the blood concentration of the limiting amino acid is depressed is left obscure, however; and one aspect of the hypothesis that required elucidation is the metabolic basis for the depression in concentration of the circulating limiting amino acid. This same paper speculated that accelerated incorporation of the limiting amino acid into protein could be the cause of its low concentration in the body fluids. Very limited data from isotopic experiments using a threonine imbalanced diet were presented, these data indicating enhanced threonine assimilation into body tissues from the imbalanced diet compared with pair-fed controls.

Recently (2) this same group has described experiments involving both threonine and histidine imbalances. These more extensive studies used isotopically labeled limiting amino acids and demonstrated more efficient retention of the limiting amino acid into liver protein, after ingestion of a single meal containing an imbalanced amino acid mixture.

Using ^{15}N-labeled histidine in diets involving a histidine imbalance we have tested the hypothesis of enhanced amino acid incorporation into tissue protein as a result of ingesting an imbalanced amino acid mixture. The resulting data, although differing in matters of detail, confirm the validity of Harper's hypothesis about the cause of the depressed concentration of the limiting amino acid in the blood.

One difference in experimental design between these experiments and those of Harper's group (1, 2) lies in the nature of the control. In the present investigation the control diet is one with the imbalance corrected by supplementation with histidine. The 2 diets are therefore almost identical in total protein level. The control diets used in Harper's experiments were the basal diets to which amino acids were added to create the imbalance. Their diet pairs consequently differ significantly in protein content. Both controls are valid, and as will be seen, both experiments lead

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to essentially the same conclusions regarding the efficiency of assimilation of the limiting amino acid from imbalanced mixtures.

EXPERIMENTAL

Eighteen weanling male rats of the Sprague-Dawley strain, weighing 40 to 45 g, were trained over an 8-day period to eat twice daily at 12-hour intervals for 2-hour periods. The basal diet during the training period consisted of the following in grams per kilogram: amino acid mixture H (3), 102.2; vitamin mixture (4), 10 (providing the following in milligrams per kilogram of ration: inositol, 1000; folacin, 2; vitamin B_{12}, 0.05; menadione, 6; niacin, 29; Ca pantothenate, 35; thiamine·HCl, 7; riboflavin, 7; pyridoxine, 14; and biotin, 0.6); salt mixture (5), 53.9; choline chloride, 2.0; corn oil, 100; α-tocopherol, 0.091; percomorph oil,[3] 0.35; and sucrose, 735.759.

An imbalanced diet with histidine limiting as described by Sauberlich (3) was prepared using the same formula except that histidine was omitted from the amino acid mixture above and replaced with 2.77 g of L-histidine-^{15}N hydrochloride/kg. The histidine contained 97 atom per cent excess ^{15}N and was uniformly labeled. To create an imbalance at the expense of sucrose 49.1 g of an amino acid mixture of the following composition was added: methionine, 4.0; leucine, 10.0; arginine·HCl, 4.0; phenylalanine, 3.75; threonine, 3.75; tryptophan, 2.5; cystine, 1.5; tyrosine, 4.0; valine, 7.0; isoleucine, 7.5; and sodium bicarbonate, 1.6. All amino acids were L-isomers, and the purity of each was confirmed on an amino acid analyzer to be 99% or greater (6). A corrected diet was also prepared identical with the imbalanced diet except that an additional 2.74 g of L-histidine were added to each kilogram of diet, this amount of histidine being adequate for complete correction of the imbalance. A portion of each diet was saved for total nitrogen and ^{15}N analyses.

At the end of the training period the 12 rats eating the most diet were selected for study and divided according to body weight into 6 groups of 2 animals each. The rats were starved for the usual 12 hours, placed in metabolism cages, and then offered 2.8 g of either the imbalanced or the corrected diet. Two, four, and six hours after feeding, 2 rats from each diet group were anesthetized and tissue samples were obtained. Actual food consumption was measured.

Blood was obtained by heart puncture and allowed tc clot. The liver, kidneys, and both gastrocnemius muscles were removed, weighed, and immediately homogenized in 10% trichloroacetic acid (TCA). The gastrointestinal tract was freed of its contents by washing with physiological saline, weighed and homogenized in 10% TCA. Stomach contents and contents of the small intestine were saved separately. Fecal and large intestine contents were pooled with the feces.

Later the liver, kidney, muscle, and gastrointestinal tract homogenates were centrifuged. The supernatants were saved, and the pellets were homogenized again in 10% TCA. After centrifuging, this supernatant was added to the first. One further washing of the pellet, this time for 30 minutes at 80 to 85°, was performed. The TCA-extracted pellets were then washed twice with acetone, dried, and weighed. Blood serum samples were deproteinized with TCA, and the pellets were washed twice with TCA, the washings being pooled with the original extracts. The carcass, left-over blood, and acetone washings of the various protein pellets were pooled and dissolved in 6 N HCl by heating at 121° for 8 hours.

Total nitrogen determinations (5) were carried out on all extracts and protein precipitates as well as on the rest of the carcass and the urine. The titrated ammonia distillates were concentrated to a 1-ml volume for ^{15}N analysis. The ^{15}N determinations followed the procedure of Burris and Wilson (7) using a Consolidated Electrodynamics Corporation Model 620-21A mass spectrometer.

A complete experiment was performed without isotope to insure the adequacy of the techniques and methodology.

RESULTS AND DISCUSSION

Table 1 summarizes the isotope intake and absorption by the 6 treatment groups. In calculating, cognizance was taken of the

[3] Oleum Percomorphum, Mead Johnson Laboratories, Evansville, Indiana.

TABLE 1

Consumption and absorption of imbalanced and corrected diets

Time after feeding	Amino acid mixture	Histidine consumed	Excess of ^{15}N consumed	Isotope absorbed as % of that eaten	Unabsorbed isotope as % of that eaten	
					Upper tract contents	Lower tract contents
hours		*μmoles*	*μmoles*			
2	Imbalanced	30.2	90.7	45.6	54.3	0.1
	Corrected	57.9	86.9	41.5	58.4	0.2
4	Imbalanced	25.6	76.7	60.1	39.7	0.2
	Corrected	49.1	73.6	59.9	39.9	0.2
6	Imbalanced	30.8	92.4	75.8	23.7	0.5
	Corrected	43.2	64.7	87.9	11.4	0.2

TABLE 2

Distribution of isotope in tissues after eating histidine-^{15}N-labeled imbalanced and corrected diets

Sample	Isotope retained, % of that absorbed					
	2 hours after feeding		4 hours after feeding		6 hours after feeding	
	Imbalanced	Corrected	Imbalanced	Corrected	Imbalanced	Corrected
Urine	0.54	1.27 *	0.28	0.39	0.69	0.83
Liver, protein	6.12	4.55	8.64	6.74 *	11.20	8.11 *
Liver, non-protein	4.09	5.29	4.18	5.58	2.21	5.76 *
Kidney, protein	0.75	0.82	1.40	1.18 *	1.55	1.30 *
Kidney, non-protein	0.15	0.34	0.34	0.34	0.19	0.36 *
GI tract, protein	7.78	5.31 *	11.36	10.93	14.69	9.33
GI tract, non-protein	5.65	4.80	3.55	4.28	2.49	2.28
Muscle,[1] protein	10.86	13.28	15.09	13.82	20.46	23.67
Muscle, non-protein	8.93	14.14	15.29	17.00	6.65	18.44 *
Serum,[2] protein	1.95	1.69	3.44	3.42	4.89	3.73 *
Serum, non-protein	3.93	0.67	1.12	1.73	0.89	1.70
Carcass	53.44	76.29	86.89	73.32	74.32	83.76
Recovery of isotope, %	80.50	100.54	118.76	104.90	109.62	114.97

* Corrected and imbalanced values significantly different at $P = 0.5$ in F test.
[1] Total muscle mass was taken as 50% of body weight.
[2] Serum volume was taken as 4% of body volume.

TABLE 3

Percentage of tissue ^{15}N isotope in protein of various tissues

Tissue	Time after feeding					
	2 hours		4 hours		6 hours	
	Imbalanced	Balanced	Imbalanced	Balanced	Imbalanced	Balanced
Liver	60	46	67	55	89	58
Kidney	80	70	80	77	89	78
GI tract	57	53	76	67	85	80
Muscle	55	48	50	42	74	54
Serum	33	70	75	66	85	68

fact that the isotope concentration of the histidine in the control diet was about half that in the experimental diet. These are average values from pairs of animals and none of the differences between imbalanced and corrected groups is statistically significant. The data confirm earlier reports (8) in that imbalanced mixtures are not poorly absorbed by the rat.

In table 2 are presented data describing the recovery of the absorbed isotope in the protein and non-protein nitrogen fractions

of various tissues as well as total isotope recovery data. The amounts of isotope excreted in the urine were extremely small, and the data are not amenable to physiological interpretation. The incorporation of isotope into protein of all tissues tends to be greater from the imbalanced mixture than from the corrected mixture as shown in table 3. With time this is increasingly so, but the trend is clear in the metabolically active tissues such as liver (see fig. 1), intestinal tract, and serum protein after 2 hours. Granted, the first statistically significant differences at the 5% level of probability occur after 4 or 6 hours, but the trends are clearly evident very early, and this point is vital if rates of conversion to protein are to be called on as explanations for the characteristic depressions in free amino acid levels in serum that come later, in this particular system after approximately 8 hours.

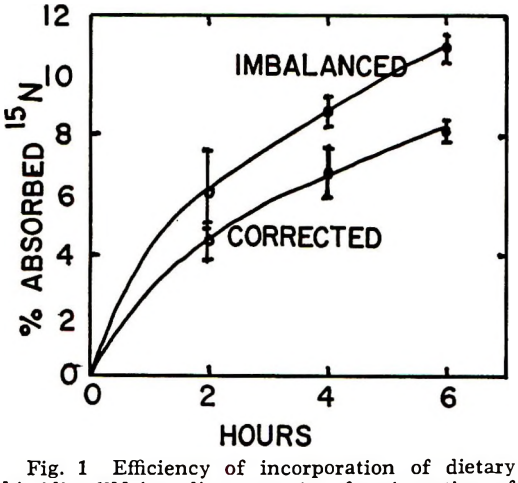

Fig. 1 Efficiency of incorporation of dietary histidine-^{15}N into liver protein after ingestion of imbalanced and corrected amino acid mixtures.

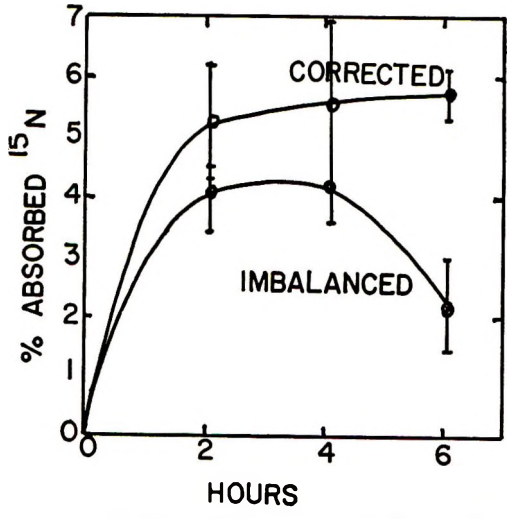

Fig. 2 Efficiency of passage of dietary histidine-^{15}N from imbalanced and corrected amino acid mixtures into the nonprotein pool of liver.

A concurrent but opposite divergence in the isotope content of the non-protein nitrogen fractions is evident in table 2 and illustrated graphically for liver in figure 2. Higher isotope levels occur in the free amino acid pools of the same tissues. This is a clear-cut trend in most tissues even after only 2 hours and is statistically significant after 6 hours in nearly all instances.

Since there was essentially no difference in the amount of isotopic histidine ingested and absorbed between the imbalanced mixture and the corrected mixture in these experiments, the direct observation of enhanced incorporation into tissue protein from the imbalanced mixture supports Harper's suggestion as to the cause of the depressed levels of free amino acids with consequent suppression of appetite.

These results confirm those of Harper's group (2) but do so independently in two important respects. The dietary formulation and feeding regimen differ, and the nitrogen rather than the carbon was labeled. In experiments such as those described here and those of Harper where analyses are carried out for isotope rather than the limiting amino acid per se, it is a necessary assurance to know the fate of both carbon and nitrogen as a safeguard against mistakenly following a metabolite of the labeled compound in the diet. To this extent the present data complement the very similar observations from the ^{14}C studies (2).

An aspect of these data which is not immediately evident is the difference in total histidine assimilation on the imbalanced and corrected regimens. The data presented in table 2 and figures 1 and 2 indicate the fraction of the absorbed histidine that is found as either protein or in the tissue pools of free amino acids. From these data it is clear that the efficiency of conversion of absorbed histidine into pro-

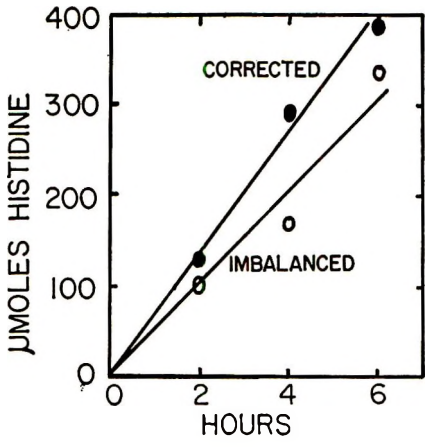

Fig. 3 Net assimilation of histidine from dietary imbalanced and corrected amino acid mixtures into liver protein.

tein is greater from the imbalanced mixture because the total histidine in the corrected diet was double that in the imbalanced diet; however, the net conversion of limiting amino acid, histidine, was greater from the corrected diet. This is shown in figure 3 in the case of liver, the other tissues showing similar behavior.

LITERATURE CITED

1. Harper, A. E., P. M-B. Leung, A. Yoshida and Q. R. Rogers 1964 Some new thoughts on amino acid imbalance. Federation Proc., 23: 1087.
2. Yoshida, A., P. M-B. Leung, Q. R. Rogers and A. E. Harper 1966 Effect of amino acid imbalance on the fate of the limiting amino acid. J. Nutr., 89: 80.
3. Sauberlich, H. E. 1961 Growth of rats fed protein-free diets supplemented with purified amino acid mixtures. J. Nutr., 74: 298.
4. King, K. W. 1963 Isotopic evaluation of efficiency of amino acid retention. Federation Proc., 22: 1115.
5. Association of Official Agricultural Chemists 1945 Official and Tentative Methods of Analysis, ed. 6, Washington, D. C., p. 27.
6. Spackman, D. H., W. H. Stein and S. Moore 1958 Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., 30: 1190.
7. Burris, R. H., and P. W. Wilson 1957 In: Methods of Enzymology, vol. 4, eds., S. P. Colowick and N. O. Kaplan, Academic Press, New York.
8. Harper, A. E. 1964 In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison. Academic Press, New York.

Control of Lipogenesis in Adipose Tissue of Fasted and Fed Meal-eating Rats [1]

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ABSTRACT The capacity of adipose tissue from meal-eating rats (animals having access to food for a single daily 2-hour period) to incorporate acetate-1-^{14}C into fatty acids in vitro was markedly stimulated by ingestion of the daily meal. Adipose tissue of fed meal-eating rats (killed after the daily meal) was found to contain more glycogen and less free fatty acids than tissue from animals killed before the daily meal (fasted). Tissue from fed animals also had a higher in vitro capacity to convert acetate-^{14}C to fatty acids, and the glycogen content of tissue from fed animals decreased by 75% during a 2-hour incubation period. These data suggest that glucose derived from glycogen was responsible for the greater lipogenic capacity of adipose tissue from fed meal-eating rats. Adipose tissue from fasted animals converted glucose-^{14}C to glyceride-glycerol at over 3 times the rate of tissue from fed meal-eaters during the first 30 minutes of incubation in vitro, whereas the conversion of glucose to fatty acids was at less than half the rate observed in tissue of fed animals. In comparison with this initial 30-minute period of incubation, the rate of glucose incorporation into glyceride-glycerol by tissue of fasted meal-eating rats decreased by about 40%, and the incorporation into fatty acids increased by approximately 270% during a subsequent 60-minute incubation period. These data suggest that lipogenesis is inhibited in adipose tissue of fasted meal-eating rats by the elevated level of free fatty acids (or fatty acyl CoA) and that glucose (supplied by ingestion of the daily meal or by addition to the incubation medium in vitro) stimulates fatty acid esterification by conversion to α-glycerophosphate, thereby lowering the level of free fatty acids and removing their inhibitory effects. In accord with this mechanism it was shown that pyruvate can stimulate in vitro fatty acid synthesis in adipose tissue of fasted meal-eating rats and that this stimulatory effect is related to the conversion of pyruvate to α-glycerophosphate. Data are also presented demonstrating that glucose is a more effective precursor of α-glycerophosphate in rat adipose tissue than is pyruvate and that glucose is apparently utilized preferentially when both substrates are available. The possible physiological significance of these observations in the meal-fed rat is discussed.

Rats trained to consume their food in a short daily period (meal-fed) develop a markedly increased capacity to convert carbohydrate to fat (1). This adaptive response is characterized by an increased lipogenic capacity of both liver and adipose tissue (2-6), but adipose tissue appears to be the major site of de novo fatty acid synthesis in the meal-fed rat (7). Accompanying the enhanced capacity for lipid synthesis in liver and adipose tissue of meal-fed rats is an increase in the activity of several enzymes related to fatty acid synthesis (4, 8–10).

These adaptive changes are evident within 5 to 11 days after the start of meal-feeding (11). However, in the adapted rat, ingestion of the daily meal per se influences lipid synthesis. Adipose tissue from meal-fed rats killed before the daily meal incorporates less acetate into fatty acids in vitro than tissue from animals killed after the daily 2-hour meal (4, 6, 12). The reasons for this difference are not obvious since the enzyme activity of adipose tissue from fasted and fed rats is similar (13) and, under the in vitro conditions of the experiment, the substrate for fatty acid synthesis, acetate, was present in excess. It has been suggested (13) that fatty acid synthesis might be inhibited in adipose tissue of fasted meal-fed rats by increased tissue levels of free fatty acids (and fatty acyl CoA derivatives) and that this

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inhibition could be removed by the availability of substrates convertible to α-glycerophosphate which would stimulate triglyceride formation. Thus, during the meal period dietary glucose could supply α-glycerophosphate and remove any inhibitory effect of tissue free fatty acids.

The experiments reported were an attempt to evaluate this hypothesis. The data presented support this proposal and suggest that free fatty acids and glycogen are important in the control of metabolism in adipose tissue of the meal-fed rat.

METHODS

Male rats of the Holtzman strain, weighing approximately 250 g, were used for all studies. The animals were housed singly in metal cages having raised wire floors, in a temperature-regulated environment (21°) and had access to food from 8 am to 10 am only (meal-eaters). Water was available at all times, and body weight and food consumption were determined at weekly intervals. The animals were maintained on this regimen for at least 3 weeks before use. This period has been shown to be of sufficient length to induce the lipogenic and enzymatic adaptations resulting from meal-feeding (11). In all experiments body weight changes and food consumption were similar to those observed previously (6). A commercial diet [2] was used in all studies and was fed in powdered form to enable measurement of food consumption.

Tissue was obtained from rats fasted for 22 hours ("fasted") or fasted for 22 hours and fed for 2 hours ("fed") unless otherwise indicated. On the day of experiment the animals were decapitated and the epididymal adipose tissue was quickly excised and treated as indicated below for the various analyses. For metabolic studies, pieces of the thin peripheral portion of the fat pad weighing approximately 100 mg were incubated in Ca^{++}-free Krebs-Ringer bicarbonate buffer (14), pH 7.4, at 38° under an atmosphere of 95% O_2-5% CO_2 for the times indicated in the tables of results. The addition of substrates and insulin [3] to the buffer is indicated in the tables of results, where appropriate. The procedures used for the isolation and counting of radioactive products were as described previously (11) except that radioactivity was determined in a Packard Tri-Carb Model 3003 liquid scintillation spectrometer. Glycogen content of adipose tissue was determined on pieces weighing from 100 to 250 mg. The tissue was extracted with chloroform:methanol (2:1), and the defatted tissue was digested in 30% KOH. The glycogen was co-precipitated with Na_2SO_4 (15), and glycogen was quantitated by the anthrone procedure (16). For free fatty acid analysis, a piece of adipose tissue weighing 250 to 500 mg was homogenized in 2 ml of H_2O in a glass homogenizer with a Teflon pestle. An aliquot of the homogenate was extracted by the method of Dole and Meinertz (17), and the fatty acids were quantitated by the colorimetric method of Mosinger (18).

RESULTS

The series of experiments to be reported were based on the hypothesis that the level of free fatty acids (or fatty acyl CoA) in adipose tissue could inhibit de novo fatty acid synthesis. The first experiment was designed to demonstrate whether ingestion of the daily meal could induce detectable changes in the level of free fatty acids in adipose tissue of meal-fed rats. The data presented in table 1 show clearly that the animals killed before the daily meal had significantly higher adipose tissue levels of free fatty acids than animals killed after the meal period. The adipose tissue glycogen content was increased in these animals as a consequence of meal ingestion. The results of this experiment suggested that the greater in vitro lipogenesis observed in tissue of fed rats as compared with fasted meal-eating rats might be related to the lower free fatty acid level and greater glycogen content of adipose tissue from fed animals.

The experiment summarized in table 2 was designed to determine whether tissue glycogen was utilized by adipose tissue incubated in vitro. There was little change in the glycogen content of adipose tissue from fasted meal-eating rats incubated with acetate-1-^{14}C, whereas the higher glycogen

[2] Rockland Mouse/Rat diet (complete), Teklad, Inc., Monmouth, Illinois.

[3] The insulin used in these studies was generously supplied by Dr. R. E. Chance, Eli Lilly and Company, Indianapolis, Indiana.

TABLE 1

Glycogen and free fatty acids in epididymal adipose tissue from fasted and fed meal-eating rats [1]

Dietary status	Adipose tissue glycogen	Adipose tissue free fatty acids
	μg/g	*mμmoles/g*
Fasted	60 ± 1 [2]	476 ± 48
Fed 1 hr	135 ± 29	—
Fed 2 hr	264 ± 119	195 ± 10

[1] Animals were killed before feeding or one or two hours after the start of the meal as indicated.
[2] Mean for 4 rats ± SE of mean.

content of tissue from fed animals decreased by approximately 75% during the 2-hour incubation period. Acetate incorporation into fatty acids was much greater in adipose tissue of fed rats as compared with fasted meal-eating animals. The addition of glucose and insulin to the incubation medium resulted in an apparent net synthesis of glycogen in tissue from fasted meal-eating rats, whereas the glycogen content of adipose tissue from fed meal-eaters decreased but to a much lesser extent than observed in the absence of glucose.

Acetate-1-^{14}C incorporation into fatty acids was somewhat similar for adipose tissue of fasted and fed meal-eating rats when incubated in the presence of glucose and insulin (table 2). However, the lower incorporation noted in this experiment for adipose tissue of fasted animals has been observed consistently under these experimental conditions. This difference between

TABLE 2

In vitro acetate-1-^{14}C incorporation into fatty acids and glycogen utilization by adipose tissue of fasted and fed meal-eating rats [1]

Dietary status	Additions to incubation medium [2]	Glycogen content	Acetate-1-^{14}C incorporation into fatty acids
		μg/g	*mμmoles/100 mg tissue/2 hr*
Fasted	initial value	37 ± 19 [3]	—
	acetate-1-^{14}C	21 ± 5	18 ± 6
	acetate-1-^{14}C + glucose + insulin	55 ± 4	336 ± 41
Fed 2 hr	initial value	447 ± 122	—
	acetate-1-^{14}C	107 ± 36	300 ± 79
	acetate-1-^{14}C + glucose + insulin	389 ± 93	546 ± 41

[1] Fasted rats were killed before the daily meal and the fed animals after the daily 2-hour meal.
[2] Both buffers contained (per ml): sodium acetate, 10 μmoles; acetate-1-^{14}C, 0.17 μCi; and, where indicated, glucose, 5 μmoles and insulin, 0.1 unit.
[3] Mean for 4 rats ± SE of mean.

TABLE 3

Rate of glucose-U-^{14}C incorporation into fatty acids and glyceride-glycerol by isolated adipose tissue of fasted and fed meal-eating rats [1]

	Meal-eating rats	
	Fasted	Fed 2 hr
	mμ moles/100 mg tissue	
Glucose-U-^{14}C incorporated into		
Fatty acids: 30-min incubation	22 ± 5 [2]	53 ± 11
90-min incubation	182 ± 32	208 ± 49
Glyceride-glycerol: 30-min incubation	59 ± 7	18 ± 2
90-min incubation	131 ± 13	56 ± 5
	mμmoles/100 mg tissue/min	
Rate of glucose-U-^{14}C incorporation into		
Fatty acids: 0–30 min	0.72 ± 0.17	1.77 ± 0.36
30–90 min	2.67 ± 0.46	2.56 ± 0.91
Glyceride-glycerol: 0–30 min	1.98 ± 0.24	0.61 ± 0.06
30–90 min	1.20 ± 0.14	0.62 ± 0.09

[1] Fasted rats were killed before the daily meal and the fed animals after the daily 2-hour meal. The buffer contained (per ml): glucose, 5 μmoles, insulin, 0.1 unit and glucose-U-^{14}C, 0.17 μCi.
[2] Mean for 5 rats ± SE of mean.

tissue of fasted and fed animals might be due to a lag in fatty acid synthesis, resulting from the higher initial level of free fatty acids in adipose tissue of fasted rats. To evaluate this possibility the experiment shown in table 3 was conducted. Adipose tissue from meal-fed rats killed before or after the daily meal was incubated in a buffer containing glucose-U-^{14}C for 30 or 90 minutes, and the incorporation of ^{14}C into fatty acids and glyceride-glycerol was determined. From these data the rates of formation of these 2 products were calculated for the first 30 minutes and the subsequent hour of incubation (table 3). Tissue from fasted meal-eaters initially incorporated much less glucose into fatty acids and more into glyceride-glycerol than tissue from fed animals. During the last hour of incubation glyceride-glycerol formation was still proceeding at a higher rate in tissue of fasted meal-eaters, but the rate of fatty acid synthesis had increased to that of tissue from fed animals. The results of this study show that there is a lag in fatty acid synthesis during the early portion of the in vitro incubation period for adipose tissue from fasted meal-eating rats.

The experiment summarized in table 4 was conducted to determine whether fatty acid synthesis might be limited in adipose tissue of fasted meal-eating rats by substrate availability. The results of this experiment (table 4) show that the oxidation of acetate-1-^{14}C by adipose tissue was not altered by the 22-hour fast, and the addition of unlabeled pyruvate to the incubation medium appeared to depress acetate oxidation slightly. The oxidation of pyruvate-2-^{14}C also was not influenced by fasting. These observations demonstrate that the ability of adipose tissue from meal-eating rats to oxidize acetate or pyruvate is not impaired by a 22-hour fast, indicating that conversion of these substrates to acetyl-CoA proceeds normally. Consequently, fatty acid synthesis in adipose tissue of fasted meal-eating animals is apparently not limited by substrate availability. The data in table 4 show that despite the apparent similarity of adipose tissue from fasted and fed meal-eaters to form acetyl CoA, there is a considerable difference in lipogenic capacity between these tissues. Adipose tissue from fasted animals incorporated much less acetate into fatty acids than did tissue from fed rats. The addition of unlabeled pyruvate stimulated acetate-1-^{14}C incorporation into fatty acids by tissue from fasted meal-eaters, but tissue from fed rats still incorporated about twice as much acetate into fatty acids. The incorporation of pyruvate-2-^{14}C into fatty acids was also higher for tissue from fed animals. The incorporation of pyruvate-2-^{14}C into glyceride-glycerol was substantial and greater in adipose tissue of fasted than fed meal-eating rats.

The data in table 5 demonstrate the effect of glucose availability on the relative utilization of specifically labeled pyruvate for fatty acid and glyceride-glycerol synthesis by adipose tissue of fasting meal-eating rats. In this experiment adipose

TABLE 4

In vitro acetate-1-^{14}C and pyruvate-2-^{14}C utilization by adipose tissue of fasted and fed meal-eating rats [1]

Incorporation of labeled substrate into	Dietary status	Additions to buffer [2]		
		Acetate-1-^{14}C	Acetate-1-^{14}C + pyruvate	Pyruvate-2-^{14}C
		mμmoles of ^{14}C-labeled substrate incorporated/100 mg tissue/3 hr		
Carbon dioxide	fasted	346 ± 31 [3]	304 ± 32	741 ± 160
	fed	349 ± 41	314 ± 54	894 ± 118
Fatty acids	fasted	10 ± 3	152 ± 42	688 ± 231
	fed	210 ± 60	252 ± 43	1412 ± 277
Glyceride-glycerol	fasted	20 ± 3	25 ± 3	342 ± 37
	fed	22 ± 3	21 ± 3	125 ± 12

[1] Fasted rats were killed before the daily meal and the fed animals after the daily 2-hour meal.
[2] All buffers contained (per ml): sodium acetate, 10 μmoles; where indicated, sodium pyruvate, 5 μmoles. Radioactive substrates were added to yield a final activity of 0.17 μCi/ml.
[3] Mean for 5 rats ± SE of mean.

TABLE 5

In vitro incorporation of specifically labeled pyruvate into fatty acids and glyceride-glycerol by adipose tissue of fasted meal-eating rats [1]

Pyruvate-^{14}C incorporation into	Radioactive substrate	Additions to buffer [2]	
		None	Glucose + insulin
		mμmoles substrate incorporated/ 100 mg tissue/3 hr	
Fatty acids	pyruvate-1-^{14}C	0.8 ± 0.1 [3]	1.2 ± 0.2
	pyruvate-2-^{14}C	709 ± 124	1595 ± 298
	pyruvate-3-^{14}C	543 ± 111	1472 ± 240
Glyceride-glycerol	pyruvate-1-^{14}C	93 ± 5	18 ± 1
	pyruvate-2-^{14}C	270 ± 17	58 ± 2
	pyruvate-3-^{14}C	210 ± 24	50 ± 4

[1] Rats were killed before the daily meal.
[2] All buffers contained (per ml): 5 μmoles pyruvate; and, where indicated, 5 μmoles glucose and 0.1 unit insulin. Radioactive substrates were added to yield a final activity of 0.17 μCi/ml.
[3] Mean for 6 rats ± SE of mean.

tissue was incubated with specifically labeled pyruvate in the absence or presence of glucose and insulin and the incorporation into fatty acids and glyceride-glycerol was determined. The addition of glucose and insulin to the incubation medium significantly decreased the conversion of pyruvate to glyceride-glycerol and increased the incorporation of pyruvate into fatty acids. This effect of glucose is similar to that of meal ingestion on pyruvate incorporation into fatty acids and glyceride-glycerol (table 4) and suggests that when glucose is available for α-glycerophosphate formation, pyruvate is diverted to acetyl-CoA and fatty acids. The incorporation patterns observed for pyruvate-1-,2-, or 3-^{14}C (table 5) are as expected (13–19). Carbon-1 of pyruvate is lost as CO_2 in the oxidation of pyruvate to acetyl-CoA and therefore should not be incorporated into fatty acids, while carbon-2 and carbon-3 should be incorporated to approximately the same extent. The incorporation of carbon-1 of pyruvate into glyceride-glycerol to about one-half the extent of carbon-2 and carbon-3 is consistent with the operation of the abbreviated dicarboxylic acid shuttle in adipose tissue (13, 19, 20).

DISCUSSION

The studies reported were undertaken in an effort to explain the stimulatory effect of meal ingestion on lipogenesis in adipose tissue of meal-eating rats. In a previous report (13) it was suggested that fatty acid synthesis might be inhibited in adipose tissue of fasted meal-eating rats by increased tissue levels of free fatty acids (and fatty acyl CoA derivatives) and that this inhibition could be removed by substrates convertible to α-glycerophosphate. This hypothesis is in accord with several reports showing that fatty acids or their acyl CoA derivatives inhibit fatty acid synthesis (21–24) and the demonstration that α-glycerophosphate can stimulate fatty acid esterification and synthesis by cell-free liver preparations (22). The data in the present report support a similar control role for free fatty acids in adipose tissue of the meal-eating rat.

In accord with the inhibitory role of tissue fatty acids, adipose tissue of fasted meal-eating rats which has a low lipogenic capacity (tables 2 and 4) also has an elevated free fatty acid level (table 1). The stimulation of lipogenesis in adipose tissue of fasted meal-eating rats by the addition of glucose (table 2) or pyruvate (table 4) to the incubation medium demonstrates that substrates convertible to α-glycerophosphate stimulate lipogenesis, presumably by esterifying fatty acids to triglycerides. Such a mechanism is also supported by the observation that glucose incorporation into fatty acids proceeds very slowly in tissue of fasted meal-eating rats during the early portion of the incubation period, whereas conversion to α-glycerophosphate is rapid (table 3). During the later stages of the incubation period, when fatty acid levels have presumably

been reduced, conversion of glucose to α-glycerophosphate or glyceride-glycerol decreases, and incorporation into fatty acids is enhanced.

The greater in vitro lipogenic capacity of adipose tissue from fed as compared with fasted meal-eating rats can be attributed to its higher glycogen and lower free fatty acid content. As demonstrated previously (11) and corroborated in the present report adipose tissue of meal-fed rats accumulates substantial quantities of glycogen during the meal period. This glycogen is utilized during in vitro incubation of adipose tissue from fed meal-eaters (table 2). The glucose derived would provide a source of α-glycerophosphate for fatty acid esterification which would maintain a low level of tissue fatty acids and enable fatty acid synthesis to proceed.

The in vitro results presented suggest a plausible regulatory mechanism which could be of physiological importance in the meal-eating rat. Adipose tissue, liver and muscle of meal-eating rats have the capacity to store unusually large quantities of glycogen during the meal period (11). Adipose tissue also accounts for over 95% of the fat synthesized during this period (7) and has the additional role of releasing fatty acids for oxidation by other tissues. Triglycerides are continuously hydrolyzed in adipose tissue and their resynthesis is dependent upon α-glycerophosphate formation from dihydroxyacetonephosphate because of the absence of glycerokinase in this tissue (25).

During the meal-period when glucose is available, little release of fatty acids would occur (26). As glucose becomes limiting, lactate, derived from glycogenolysis and glycolysis in muscle (27), could serve as a source of α-glycerophosphate in adipose tissue after conversion to pyruvate (12, 19, 20, 28) and for a time could limit fatty acid release (29). As both glucose and lactate become limiting, tissue fatty acid levels would increase, lipogenesis would be curtailed and fatty acids would be released rapidly. Such a regulatory mechanism is consistent with the in vitro observations reported in the present paper and recent studies of the effect of fatty acids on lipogenesis (21–24) and enzyme activity (30) in liver. Studies in progress will attempt to evaluate the significance of this mechanism in the intact meal-fed rat.

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LITERATURE CITED

1. Tepperman, J., J. R. Brobeck and C. N. H. Long 1943 The effects of hypothalamic hyperphagia and of alterations in feeding habits on the metabolism of the albino rat. Yale J. Biol. Med., *15:* 875.
2. Tepperman, J., and H. M. Tepperman 1958 Effects of antecedent food intake pattern on hepatic lipogenesis. Amer. J. Physiol., *193:* 55.
3. Cohn, C., and D. Joseph 1960 Effects on metabolism produced by the rate of ingestion of the diet. "Meal-eating" versus "nibbling". Amer. J. Clin. Nutr., *8:* 682.
4. Hollifield, G., and W. Parson 1962 Metabolic adaptations to a "stuff and starve" feeding program. I. Studies of adipose tissue and liver glycogen in rats limited to a short daily feeding period. J. Clin. Invest., *41:* 245.
5. Stevenson, J. A. F., V. Feleki, A. J. Szlavko and J. R. Beaton 1964 Food restriction and lipogenesis in the rat. Proc. Soc. Exp. Biol. Med., *116:* 178.
6. Leveille, G. A., and R. W. Hanson 1965 Influence of periodicity of eating on adipose tissue metabolism in the rat. Can. J. Physiol. Pharmacol., *43:* 857.
7. Leveille, G. A. 1967 In vivo fatty acid synthesis in adipose tissue and liver of meal-fed rats. Proc. Soc. Exp. Biol. Med., in press.
8. Tepperman, H. M., and J. Tepperman 1958 The hexosemonophosphate shunt and adaptive hyperlipogenesis. Diabetes, *7:* 478.
9. Tepperman, H. M., and J. Tepperman 1964 Patterns of dietary and hormonal induction of certain NADP-linked liver enzymes. Amer. J. Physiol., *206:* 357.
10. Leveille, G. A., and R. W. Hanson 1966 Adaptive changes in enzyme activity and metabolic pathways in adipose tissue from meal-fed rats. J. Lipid Res., *7:* 46.
11. Leveille, G. A. 1966 Glycogen metabolism in meal-fed rats and chicks and the time sequence of lipogenic and enzymatic adaptive changes. J. Nutr., *90:* 449.
12. Hollifield, G., and W. Parson 1965 Effect of feeding on fatty acid synthesis. In: Handbook of Physiology, section 5, eds., A. E. Renold and G. F. Cahill, Jr. American Physiological Society, Washington, D. C., p. 393.
13. Leveille, G. A. 1967 Lipogenesis in adipose tissue of meal-fed rats. A possible regulatory role of α-glycerophosphate formation. Can. J. Physiol. Pharmacol., in press.
14. Umbreit, W. W., R. H. Harris and J. F. Stauffer 1964 Manometric Techniques, ed. 4.

Burgess Publishing Company, Minneapolis, p. 132.
15. Van Handel, E. 1965 Estimation of glycogen in small amounts of tissue. Anal. Biochem., *11:* 256.
16. Hassid, W. Z., and S. Abraham 1957 In: Methods in Enzymology, vol. 3, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 34.
17. Dole, V. P., and H. Meinertz 1960 Microdetermination of long-chain fatty acids in plasma and tissues. J. Biol. Chem., 235: 2595.
18. Mosinger, F. 1965 Photometric adaptation of Dole's microdetermination of free fatty acids. J. Lipid Res., 6: 157.
19. Ballard, F. J., G. A. Leveille and R. W. Hanson 1967 Phosphoenolpyruvate carboxykinase and the synthesis of glyceride-glycerol from pyruvate in adipose tissue. J. Biol. Chem., in press.
20. Leveille, G. A. 1967 The reversal of glycolysis in rat epididymal adipose tissue. Life Sci., in press.
21. Tubbs, P. K., and P. B. Garland 1964 Variations in tissue contents of coenzyme A thio esters and possible metabolic implications. Biochem. J., *93:* 550.
22. Howard, C. F., Jr., and J. M. Lowenstein 1965 The effect of glycerol 3-phosphate on fatty acid synthesis. J. Biol. Chem., 240: 4170.
23. Cahill, G. F., Jr., B. Leboeuf and R. B. Flinn 1960 Studies on rat adipose tissue in vitro. VI. Effect of epinephrine on glucose metabolism. J. Biol. Chem., 235: 1246.
24. Korchak, H. M., and E. J. Masoro 1964 Free fatty acids as lipogenic inhibitors. Biochim. Biophys. Acta, 84: 750.
25. Vaughan, M. 1961 The metabolism of adipose tissue in vitro. J. Lipid Res., 2: 293.
26. Vaughan, M. 1962 The production and release of glycerol by adipose tissue incubated in vitro. J. Biol. Chem., 237: 3354.
27. Helmreich, E., and C. F. Cori 1965 Regulation of glycolysis in muscle. Advance. Enzyme Regulat., 3: 91.
28. Leveille, G. A. 1966 *In vitro* lipogenesis in adipose tissue of fed and fasted rats: Mechanism of pyruvate stimulation. Life Sci., 5: 421.
29. Jungas, R. L., and E. G. Ball 1963 Studies on the metabolism of adipose tissue. XII. The effects of insulin and epinephrine on free fatty acid and glycerol production in the presence and absence of glucose. Biochemistry, 2: 383.
30. Weber, G., H. J. H. Covery, M. A. Lea and N. B. Stamm 1966 Feedback inhibition of key glycolytic enzymes in liver: Action of free fatty acids. Science, *154:* 1357.

Vitamin A, Sulfation and Bone Growth in the Chick [1]

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ABSTRACT The enzyme ATP-sulfurylase, though present in chick liver, brain and spinal cord, was not affected by vitamin A deficiency. However, the vitamin deficiency caused increased organic (but not inorganic) deposition in chick bone, increased water content, and increased total wet weight of bones. Chondroitin sulfate, as measured by uronic acid concentration and sulfate uptake, was higher in the epiphyses of deficient bone. Tricarboxylic acid cycle and glycolysis reactions and uptake of amino acids into protein took place at a higher rate in deficient bone. No differences were found for collagen formation. These results tend to suggest that the increased metabolic activity of deficient bone may accompany some form of overgrowth of the deficient bones, possibly of the intercellular matrix.

The hypothesis was proposed by one of us (1) that vitamin A functions in the reaction leading to the activation of the inorganic sulfate ion. The experiments needed to demonstrate this involved assay of the enzyme ATP-sulfurylase in normal and vitamin A-deficient rats. However, it was soon found that starvation also causes a severe lowering of this enzyme. [3] Therefore, the question arose: Is the decrease in activity of the sulfurylase in vitamin A deficiency solely the effect of the lowered food intake attendant upon the vitamin deficiency, or has the vitamin deficiency an effect on the enzyme in addition to that of starvation?

It was suggested [4] that we investigate the effect of vitamin A deficiency on the activity of sulfurylase in the chick since in this species, as opposed to the rat, food intake in normal and deficient animals is identical. This study was carried out. No difference was found in the liver sulfurylase between normal and vitamin A-deficient chicks. We then argued that since the only observable symptom of the vitamin deficiency in the chick involves the nerves, the first lesion due to the deficiency is to be sought in the nervous system. However, the sulfurylase, though present in brain and spinal cord, was not lowered in its activity by vitamin A deficiency.

The controversy with respect to the nervous lesions accompanying vitamin A deficiency, especially in birds, has not been settled. The following hypotheses have been proposed: (a) an effect of the vitamin deficiency on the nerve tissue directly; (b) an effect of compression of the central nervous system by its normal growth within a skull and vertebral column which is not growing normally; (c) an effect of overgrowth of skull bones and thereby compression of the central nervous system; (d) compression of the nerve tissue by increase in cerebrospinal fluid pressure found to accompany vitamin A deficiency.

As we found at least one enzyme system of nerve tissue, previously thought to be connected with vitamin A function, not affected by the deficiency, we have attempted to throw some light on hypotheses (b) and (c). We have studied some of the biochemical parameters of bone, especially of the skull, in the vitamin A-deficient chick. A similar study (2), carried out purely histologically, demonstrated that in the chick the nervous symptoms are due to bone growth retardation.

EXPERIMENTAL

Preparation of vitamin A-deficient chicks. One-day-old chicks [5] were fed the deficient diet (table 1). Control chicks

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[2] On leave of absence from the Department of Nutrition, The Hebrew University, Hadassah Medical School. Jerusalem, Israel.

[3] Root, D. M. 1966 Studies on the relation of ATP-sulfurylase to starvation and vitamin A deficiency. Thesis, M.I.T.

[4] Suggestion made by Prof. R. A. Morton.

[5] Leghorn, from Cobbs' Pedigree Chicks, Littleton, Massachusetts.

TABLE 1

Vitamin A-deficient diet for the chick

	%
Casein (vitamin-free)	35
Glucose	40.6
Cellulose	3.0
Salt mix [1]	5.0
Arginine	1.2
DL-Methionine	0.5
Calcium carbonate	1.0
Glycine	1.5
Choline chloride	0.2
Cottonseed oil	10.0
Vitamin mix (vitamin A-free) [2]	2.0

[1] According to H. Spector 1948 J. Biol. Chem.,173: 659.

[2] Vitamin A-free mix: (in g) thiamine, 1.0; riboflavin, 1.0; pyridoxine, 1.0; Ca pantothenate, 2.0; niacin, 1.5; inositol, 2.5; folic acid, 0.1; vitamin E acetate, 10.0; biotin, 0.0075; menadione, 2.0; vitamin D_3, 125,000 IU; and sucrose to make 1.000 g.

were given water-soluble vitamin A [6] (300 IU/100 g feed) every 48 hours. The deficient birds became ataxic between day 15 and 18, at which time they were used for the experiments.

Assay of ATP-sulfurylase. Male chicks weighing between 290 and 350 g were decapitated. The livers were excised, cleaned with ice-cold saline, homogenized and separated into particulate and supernatant fractions by centrifugation as described by Sundaresan (3). The ATP-sulfurylase was assayed in the supernatant fraction by the reaction of ATP with sodium molybdate with assay of the pyrophosphate generated, as worked out in this laboratory (3). As an independent check on the method, the samples were also assayed by reaction of ATP with labeled sodium sulfate, conversion of the adenosine phosphosulfate formed to phosphoadenosine phosphosulfate by the kinase present in the supernatant solution, and transfer of the activated sulfate thus formed to *p*-nitrophenol by addition of a preparation of sulfate transferase. The resultant labeled *p*-nitrophenophenylsulfate was separated by paper chromatography and assayed.[7]

Preparation and incubation of bone tissue. Male chicks weighing between 290 and 350 g were decapitated. The skull and tibia were removed after careful separation through the distal epiphyseal plate. All adherent connective tissue, including the cartilage of the head, was removed. Each bone was dissected, cleaned with filter paper and rinsed with 0.9% sodium chloride solution. The epiphyses and diaphyses were then cut into thin slices with a sharp scalpel. The small pieces of bone (200–400 mg) were weighed and immediately placed in 10-ml Erlenmeyer flasks containing 3 ml of Krebs-Ringer bicarbonate buffer, ph 7.4, glucose 0.17 mM, and small amounts of penicillin and streptomycin. A stream of 95% O_2-5% CO_2 was bubbled through the medium for 1 minute, and the flasks were stoppered and incubated at 37° in a Dubnoff shaker at 70 oscillations/minute for 3 hours. For blank experiments, the bone slices were placed in a boiling water bath for 5 minutes before incubation.

After incubation, the medium was removed, and the bone fragments were decalcified by 2 exposures to 10 ml of a 10% solution of EDTA for 48 hours each. The decalcified material was washed 3 times with water.

Determination of radioactivity in CO_2 liberated. Incubations were carried out as above in Erlenmeyer flasks containing a center well and stoppered by a rubber diaphragm. At the end of the incubation, the flasks were chilled in ice, 0.5 ml 1 M Hyamine hydroxide in methanol [8] was injected into the center well by needle and syringe followed by 0.3 ml 1 N sulfuric acid into the medium. The flasks were then shaken for an additional hour to complete absorption of the $^{14}CO_2$. The Hyamine solution was then transferred to a scintillation vial containing 6 ml ethanol and 8 ml toluene with 0.6% scintillator (DPO). Counting efficiency in a liquid scintillation spectrometer [9] was 67%.

Determination of hydroxyproline. The decalcified bone slices were hydrolyzed in 2 ml 6 N HCl in sealed tubes at 110° for 24 hours. The specific activity of the hydroxyproline was determined by method of Prockop and Udenfriend (4).

Determination of proline and leucine incorporation into matrix protein. Aliquots of the decalcified matrix, hydrolyzed as above, were counted in the ethanol-toluene system described. Protein nitrogen was determined by the Kjeldahl-Nessler method.

[6] Mann Research Laboratories, New York. Vitamin A palmitate (dry, water-dispersible), 250,000 USP units/g.

[7] Personal communication, Dr. J. Carrol.

[8] Packard Instrument Company, LaGrange, Illinois.

[9] Nuclear Chicago Corporation, Des Plaines, Illinois.

Determination of uronic acid and sulfate. The decalcified matrix was hydrolyzed in 2 ml acetate buffer at pH 5.5 containing papain (0.1 ml/g of tissue; 2 × crystallized papain suspended in 0.05 M sodium acetate) [10] for 24 hours at 65°. At the end of this time, the incubations were placed in boiling water for 1 minute and dialyzed for 72 hours against distilled water (10 liters, changed 3 times).

Radioactivity of the sulfate in the chondroitin sulfate was determined by placing aliquots from the dialysis bag contents in the scintillation solution described. The amount and radioactivity of uronic acid was measured by the method of Bitter and Muir (5).

Determination of calcium. Calcium-45 was determined according to the procedure of Carr and Parsons (6); calcium in bone ash by the method of Baron and Bell (7).

RESULTS

Figure 1 shows the growth curves for vitamin A-deficient and normal chicks. At least up to day 18 when the animals showed severe ataxia, there was no difference in growth rate and hence, in food intake. After day 18 deficient birds began to show a decline in growth, but it appeared to be mostly because of their inability to walk and therefore to get to the feed. No

[10] Sigma Corporation, St. Louis.

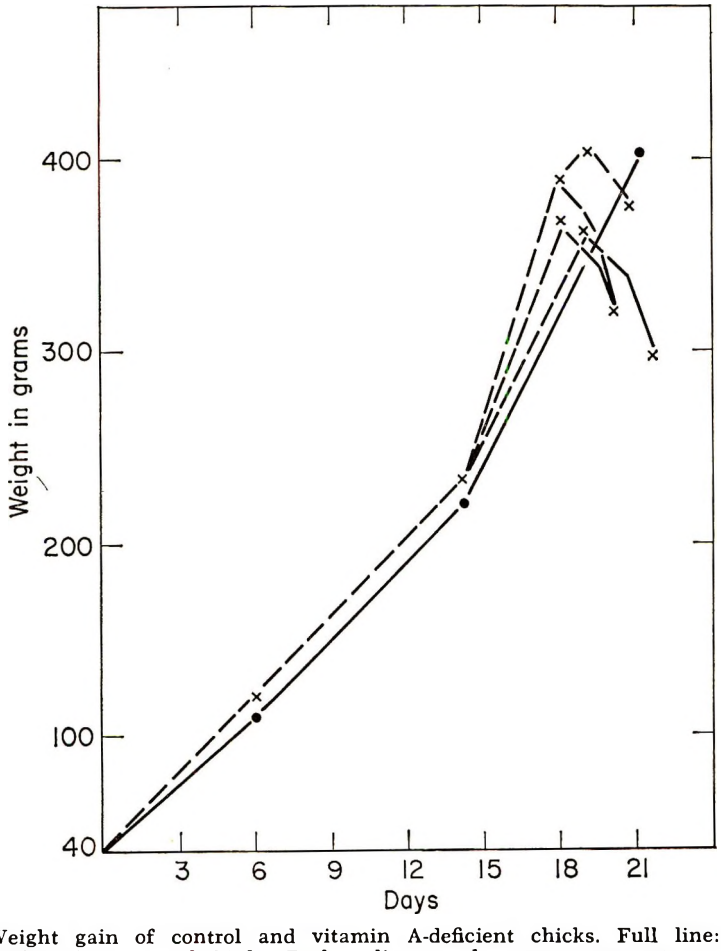

Fig. 1 Weight gain of control and vitamin A-deficient chicks. Full line: represents average weight of 6 control birds. Broken line: each curve represents weights of 1 deficient bird.

experiments were performed on chicks after day 18.

The data in table 2 demonstrate that though there was some ATP-sulfurylase activity present in chick liver, brain and spinal cord, it was not affected by the vitamin deficiency.

With respect to bone growth, cursory inspection showed the bones of the deficient birds to be longer and wider, the cortical bone to be greater in width, and the amount of spongeous bone in the epiphysis to be greater. Table 3 shows these effects in terms of chemical analysis: deficient bone, but especially that of the skull, has a higher percentage of nitrogen and a very much larger amount of water than normal bone. It is clear therefore that increased bone size (table 3) is not due to increased deposition of inorganic material (column 3, table 3). In terms of chondroitin sulfate, also, as measured by uronic acid level, at least in epiphyseal bone, there is an increase in the deficient bird (table 3).

From such static measurements of the amount of substances present, we next turned to dynamic experiments, in which the metabolism and incorporation of substrates in bone were studied.

Table 4 shows that for epiphyseal and calvarial bone, the energy-yielding reactions of glycolysis and the tricarboxylic acid cycle take place at a higher rate in the deficient as compared with normal bone. As for incorporation of radioactive amino acids into the bone matrix protein (table

TABLE 2

Determination of ATP-sulfurylase activity in the chick

	PP formed/hr/mg protein [1]
	μmoles
Liver	
Control	1.04 ± 0.15 [2,3]
Vitamin A-deficient	1.42 ± 0.17 [3]
Boiled enzyme	0.07 ± 0.01 [3]
Brain	
Control	0.52 ± 0.08 [4]
Vitamin A-deficient	0.51 ± 0.12 [4]
Spinal cord	
Control	0.63 ± 0.07 [4]
Vitamin A-deficient	0.65 ± 0.08 [4]

[1] Each value is the mean of 6 incubations.
[2] SE of mean.
[3] *P* value < 0.1.
[4] *P* value, not significant.

TABLE 3

Chemical composition of chick bones in vitamin A deficiency

	Wet wt of fresh bone	Wt of dry bone	Ash	Total N	Ash/N ratio	Uronic acid
	mg	*% of fresh bone*	*% of dry bone*	*% of dry bone*		*μg/g fresh bone*
Epiphyses (tibia)						
Control	1072 ± 61.7 [1,2]	49.0 ± 5.1 [3]	52.05 ± 2.9 [4]	6.59 ± 0.43	8.0	534 ± 72 [3]
Vitamin A-deficient	1222 ± 78.9 [2]	35.6 ± 3.4 [3]	52.8 ± 5.0 [4]	7.87 ± 0.51	6.7	1154 ± 127 [3]
Diaphyses (tibia)						
Control	743 ± 63.8 [2]	69.3 ± 6.1 [4]	67.6 ± 3.1 [4]	5.16 ± 0.28	13.1	487 ± 66 [4]
Vitamin A-deficient	1055 ± 47.6 [2]	62.5 ± 5.0 [4]	66.8 ± 7.8 [4]	6.50 ± 0.36	10.3	524 ± 88 [4]
Calvaria (skull)						
Control	469 ± 24.7 [5]	54.6 ± 4.5 [3]	55.7 ± 8.3 [4]	6.09 ± 0.36	9.1	478 ± 72 [4]
Vitamin A-deficient	491 ± 35.3 [5]	39.9 ± 5.7 [3]	53.9 ± 2.9 [4]	8.75 ± 0.27	6.1	459 ± 81 [4]

[1] SE of mean; number of control chicks, 36; number of deficient chicks, 24.
[2] *P* value << 0.001.
[3] *P* value << 0.01.
[4] *P* value, not significant.
[5] *P* value < 0.025.

TABLE 4

Isotope experiments with bones of vitamin A-deficient and normal chicks in vitro

	Epiphyses (tibia)		Diaphyses (tibia)		Calvaria (skull)	
	Deficient	Control	Deficient	Control	Deficient	Control
	dpm × 10^3	*dpm* × 10^3	*dpm* × 10^3	*dpm* × 10^3	*dpm* × 10^3	*dpm* × 10^3
Glucose-1-^{14}C [1] conversion to $^{14}CO_2$ by bone	19.6 ± 1.9 [2,3]	10.2 ± 1.3 [3]	4.85 ± 0.95 [4]	3.93 ± 0.55 [4]	7.30 ± 0.54 [3]	6.00 ± 0.59 [3]
Sodium acetate-1-^{14}C [5] conversion to $^{14}CO_2$ by bone	643.8 ± 47.3 [3]	467.7 ± 32.1 [3]	266.0 ± 38.0 [3]	148.2 ± 8.8 [3]	733.6 ± 76.0 [3]	266.3 ± 29.4 [3]
Leucine-1-^{14}C [6] incorporation into bone matrix protein	293.0 ± 57 [3]	189.5 ± 29 [3]	135.0 ± 39.3 [3]	71.0 ± 8.6 [3]	283.4 ± 34.7 [7]	206.2 ± 37.5 [7]
Sulfate-35 [8] incorporation into bone matrix	12.1 ± 1.9 [3]	5.4 ± 0.45 [3]	4.8 ± 0.70 [4]	4.0 ± 0.48 [4]	9.4 ± 0.84 [4]	8.8 ± 0.90 [4]
Proline-^{14}C [6] incorporation into bone matrix	96.0 ± 5.0 [3]	56.9 ± 3.5 [3]	37.8 ± 3.7 [4]	38.6 ± 3.2 [4]	68.7 ± 4.0 [4]	72.4 ± 4.3 [4]
Proline-^{14}C [6] incorporation into hydroxyproline of bone matrix	14.7 ± 1.23 [4]	15.1 ± 1.0 [4]	3.9 ± 0.24 [4]	4.4 ± 0.45 [4]	19.8 ± 1.6 [4]	20.0 ± 1.8 [4]
Hydroxyproline of bone matrix, mg/g bone	42.7	41.2	43.6	38.7	47.3	46.2
Hydroxyproline of bone matrix, specific activity, dpm/mg	345	370	90	112	420	433

[1] 1 μCi; specific activity, 100 μCi/mmole; all isotopes were obtained from New England Nuclear Corporation, Boston.
[2] SE of mean.
[3] *P* value < 0.01.
[4] *P* value, not significant.
[5] 1 μCi; specific activity, 2 mCi/mmole.
[6] 1 μCi; specific activity, 200 μCi/mmole.
[7] *P* value, < 0.02.
[8] 1 μCi; specific activity, 10 mCi/mmole.

TABLE 5

Calcium in bones of normal and vitamin A-deficient chicks [1]

	Ca in ash	Radioactivity	Specific activity
	%	*dpm* × 10^3/*g ash*	*dpm* × 10^3/*mg Ca*
Epiphyses (tibia)			
Control	34.8 ± 1.7 [2,3]	4692 ± 127 [4]	13.7
Vitamin A-deficient	38.3 ± 1.2 [3]	5144 ± 143 [4]	13.4
Diaphyses (tibia)			
Control	36.7 ± 1.8 [5]	2968 ± 198 [5]	8.3
Vitamin A-deficient	36.9 ± 1.7 [5]	2782 ± 137 [5]	7.5
Calvaria (skull)			
Control	35.0 ± 1.9 [5]	4304 ± 143 [5]	12.3
Vitamin A-deficient	32.5 ± 0.9 [5]	4143 ± 125 [5]	12.8

[1] $^{45}CaCl_2$ was injected intraperitoneally (1 μCi/35 g body weight; specific activity, 4 mCi/mg) (New England Nuclear Corporation, Boston). Animals killed after 18 hours.
[2] SE of mean.
[3] *P* value < 0.01.
[4] *P* value < 0.02.
[5] *P* value, not significant.

4), there appeared to be a somewhat greater rate of this process in the deficient bone compared with normal, in keeping with the greater percentage of nitrogen found in deficient bone. As for collagen, as measured by hydroxyproline (table 4), neither total nor specific activity showed any difference between the 2 types of bird.

Labeled sulfate was incorporated to a greater extent into deficient bone, at least in epiphysis (table 4), in which type of bone there was also a slight increase in calcium incorporation (table 5).

DISCUSSION

The present results strongly suggest that, at least in the chick, the enzyme ATP-sulfurylase is not involved in the metabolic function of viatmin A. There may still be such a function in the rat, and in fact even in the chick after the nervous symptoms have appeared. However, since the birds died shortly after the appearance of the nervous symptoms, this possibility has not been tested. It should also be borne in mind that there are species differences in the response to vitamin A; whereas Morton and Phillips (8) found a great increase in liver ubiquinone in vitamin A-deficient rats, this co-factor did not increase in deficient chicks (9). This might also account for the fact that De Luca and Wolf [11] found a decrease in sulfate incorporation into purified chondroitin sulfate of rat cartilage in vitamin A deficiency, whereas in the present work, we observed an increase, at least in epiphyseal bone.

Lucy et al. (10), working with chick embryo bone in organ culture in the presence of excess vitamin A, demonstrated a decreased amount of protein and chondroitin sulfate in the bone matrix. It might therefore be expected that the opposite effect, an increase in protein and mucopolysaccharide of chick bone, would take place under conditions of the vitamin deficiency. This increase was actually observed in the present work. However, this interpretation would not be in agreement with observations by Dingle et al. (11) and Roels et al. (12) who showed that at least in rat liver, vitamin A excess *and* vitamin A deficiency result in increased release of lysosomal enzymes. It is this release which is responsible for the dissolution of chick embryo bone (13).

However, a recent report by Wolke [12] demonstrates a decrease in epiphyseal bone tissue and reduction in bone length and weight, all due to decreased osteoblastic activity taking place as a result of *hyper*vitaminosis A in the pig. Moreover, this author found an increased bone-ash-to-nitrogen ratio and decreased organic matter, both truly the opposite of our findings in *hypo*vitaminosis A.

The increase in oxidation of acetate in vitamin A-deficient bone is in agreement with the data of De Luca et al. (14) who noted greater tricarboxylic acid oxidation

[11] Unpublished results.
[12] Wolke, R. E. 1966 The osseous pathology of porcine hypervitaminosis A. Thesis. University of Connecticut.

activity in mitochondria of vitamin A-deficient rat liver.

The results of the present work were somewhat unexpected in view of the precise and well-documented demonstration by Wolbach and Hegsted (2) that vitamin A deficiency causes retardation of bone growth in chicks and in view of the hypothesis of one of us that the vitamin deficiency decreases sulfated mucopolysaccharide synthesis (1), as was in fact observed in the rat.[13] The present results of increased weights, water content and nitrogen content of deficient bones would point to *increased* bone cells or matrix. There is no doubt that the deficient bones have increased metabolic activity, a phenomenon generally associated with growth. Perhaps the discrepancy might be explained by postulating retardation of bone growth at the same time as an increase of intercellular matrix. However, at least in the epiphyses of tibia, we observed a slight increase in calcification caused by the deficiency.

It appears that the very clear-cut histological findings of Wolbach and Hegsted (2), demonstrating that the cause of nervous symptoms is compression due to bone growth retardation, may have to be modified by biochemical findings not incompatible with some form of bone overgrowth.

LITERATURE CITED

1. Varandani, P. T., G. Wolf and B. C. Johnson 1960 Function of vitamin A in the synthesis of 3′-phosphoadenosine-5′-phosphosulfate. Biochem. Biophys. Res. Commun., *3:* 97.
2. Wolbach, S. B., and D. M. Hegsted 1952 Vitamin A deficiency in the chick. Arch. Pathol., *54:* 13.
3. Sundaresan, P. R. 1966 Vitamin A and the sulfate-activating enzymes. Biochim. Biophys. Acta, *113:* 95.
4. Prockop, D. J., and S. Udenfriend 1960 A specific method for the analysis of hydroxyproline in tissues and urine. Anal. Biochem., *1:* 228.
5. Bitter, J., and H. M. Muir 1962 A modified uronic acid carbazole reaction. Anal. Biochem., *4:* 330.
6. Carr, T. E. F., and B. J. Parsons 1962 A method for the assay of calcium-45 by liquid scintillation counting. Int. J. Appl. Radiat. Isotop., *13:* 57.
7. Baron, D. N., and S. Bell 1959 Complexiметric determination of calcium in pathological and physiological specimens. J. Clin. Pathol., *12:* 143.
8. Morton, R. A., and W. E. J. Phillips 1959 Vitamin A deficiency and the ubiquinone and substance SC contents of rat liver: The time factor. Biochem. J., *73:* 416.
9. Lowe, J. S., R. A. Morton, N. F. Cunningham and J. Vernon 1957 Vitamin A deficiency in the domestic fowl. Biochem. J., *67:* 215.
10. Lucy, J. A., J. T. Dingle and H. B. Fell 1961 Studies on the mode of action of excess vitamin A. Biochem. J., *79:* 500.
11. Dingle, J. T., I. M. Sharman and T. Moore 1966 Nutrition and lysosomal activity. Biochem. J., *98:* 476.
12. Roels, O. A., M. Trout and A. Guha 1965 The effect of vitamin A deficiency and dietary alpha-tocopherol on the stability of rat-liver lysosomes. Biochem. J., *97:* 353.
13. Dingle, J. T. 1961 Studies on the mode of action of excess vitamin A. Biochem. J., *79:* 509.
14. DeLuca, H. F., M. R. Manatt, N. Madsen and E. B. Olson 1963 Action of vitamin A on liver homogenate oxidation of tricarboxylic acid cycle intermediates. J. Nutr., *31:* 383.

[13] Unpublished results.

Effect of Low Environmental Temperature on the Metabolism of Vitamin A (Retinol) [1] in the Rat

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ABSTRACT The effects of low environmental temperature on liver vitamin A utilization of rats were examined after two, four and six weeks of exposure at 5°. Total liver vitamin A levels were unchanged. The weight gain of animals at 5° was always less than the weight gain at 25°. An increased utilization of vitamin A was indicated if the utilization of the vitamin was expressed as a ratio of the amount of vitamin A removed from the liver to the weight gain of the animal. The increase in the vitamin A depletion ratio observed in the 4-week cold-exposed rats was abolished by administration of thiouracil at a level of 0.1% in the diet. An increased requirement for vitamin A in the cold was indicated by the reduced survival time of vitamin A-deficient rats exposed to cold. In addition, at least 20 times more retinoic acid was necessary to maintain growth and survival in the cold than at 25°.

A recent study by Phillips (1) indicated that the hepatic storage and the rate of metabolism of orally administered retinyl acetate were unaffected by environmental temperature, thus suggesting that prolonged exposure to cold did not increase the vitamin A requirement. This was in contrast with the earlier studies of Ershoff (2) who noted decreased survival times of vitamin A-deficient rats at low environmental temperatures (3°) and, accordingly, concluded that the vitamin A requirement was increased by prolonged exposure to cold. In a subsequent study on the effect of graded doses of retinyl palmitate on the resistance to low environmental temperatures, Ershoff (3) reported that an average daily intake of 0.9 μg of retinyl palmitate, while sufficient for growth and survival under room temperature conditions, was not adequate for maintenance in the cold (2°). More recently, Porter and Masoro (4) showed that cold-acclimated rats stored less vitamin A in the liver than rats acclimated to 25° when fed a diet low in vitamin A with weekly supplementation of vitamin A.

The increased metabolic activity observed during cold stress suggests a possible interrelationship of the thyroid and vitamin A utilization which has been studied in the rat by several investigators. Johnson and Baumann (5) observed that after feeding carotene orally, liver storage of vitamin A was depressed in hypothyroidism and increased in hyperthyroidism. Under similar conditions, no effect was observed when preformed vitamin A was fed. In a subsequent study, however, Johnson and Baumann (6) showed that the depletion of vitamin A from the liver depended not only upon the basal metabolic rate but also upon the growth rate. Restricted growth had a more pronounced effect on retarding the depletion of liver vitamin A than increased thyroid activity had on increasing the depletion of vitamin A.

In an attempt to resolve some of the conflicting reports, the effect of cold exposure on vitamin A utilization was investigated. In view of the possible thyroid interrelationship, the effect of feeding thiouracil at a level of 0.1% in the diet (6) on vitamin A utilization in the cold was also studied. Since retinoic acid has been shown to possess high biological activity without being reduced to retinol in vivo (7), a current view is that retinoic acid is the precursor of, or is itself, the system-

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[1] The specific terms retinol, retinyl acetate and retinoic acid are used in place of vitamin A alcohol, vitamin A acetate and vitamin A acid, respectively. The term vitamin A, when it appears, is used to indicate total retinyl esters and retinol.

[2] This investigation was carried out while P. R. Sundaresan was a National Academy of Sciences — National Research Council Visiting Scientist at the U. S. Army Research Institute of Environmental Medicine.

ically "active" form of vitamin A (8, 9). Accordingly, in survival studies on cold exposure, graded doses of retinoic acid were administered to vitamin A-deficient rats.

METHODS AND MATERIALS

Male, weanling albino rats of the Holtzmann strain, weighing between 40 to 50 g, were fed ad libitum throughout all experiments a purified vitamin A free-diet (table 1). After approximately 4 weeks, when the rats had reached a plateau in weight (200–240 g), retinyl acetate in an oil solution was administered orally (0.1 ml) by syringe daily until a total of about 2200 μg of retinyl acetate had been given. The rats were then divided into 3 groups. One group served as zero-time controls and was killed at the beginning of the experimental period. Another group was kept at 5°, and the remaining group at 25°. Three experiments were conducted with this design. In experiment 1, one-half of the rats from both experimental groups were killed at the end of 2 weeks. The remainder were killed at the end of 4 weeks. In experiment 2, the animals in both experimental groups were killed at the end of 6 weeks. In experiment 3, the rats in both groups, after receiving the vitamin A-free diet for 2 weeks, were fed 0.1% thiouracil incorporated into the vitamin A-free diet. After 2 weeks of thiouracil-feeding, the rats were killed.

TABLE 1

Composition of the vitamin A-free diet [1] used in the different experiments

	%
Casein [2] (alcohol-extracted)	18
Purified dextrin [3]	62.8
Sucrose	10.0
Cottonseed oil [4]	5.0
Salt mixture 446 [5]	4.0
Choline chloride	0.2

[1] The vitamin A-free diet also contained the following vitamins per kilogram of diet: (in milligrams) thiamine·HCl, 3; riboflavin, 15; niacin, 100; Ca pantothenate, 40; pyridoxine·HCl, 6.0; biotin, 0.6; folic acid, 4.0; menadione, 4.0; vitamin B_{12} (0.1% triturated), 50; *i*-inositol, 20; *p*-aminobenzoic acid, 6; vitamin D_2 (400,000 IU/g), 6.2; α-tocopheryl acetate beadlets (25%), 600.5; and ascorbic acid 1000 (15, 16).

[2] Vitamin-Free Test Casein (General Biochemicals, Inc., Chagrin Falls, Ohio) was extracted with acidified ethanol for 24 hours and dried at 50° for 6 hours before use.

[3] Obtained from General Biochemicals, Inc.

[4] Wesson Oil, Wesson Sales Company, Fullerton, California.

[5] Each 100 g of the salt mixture supplied: (in grams) sodium chloride, 10.8088; potassium citrate, 23.6888; potassium phosphate (dibasic), 7.7332; calcium phosphate (dibasic), 35.5554; calcium carbonate, 16.3554; magnesium carbonate, 4.0888; iron citrate, 1.5998; copper sulfate, 0.0178; manganous sulfate, 0.1245; potassium aluminum sulfate, 0.0009; potassium iodide, 0.0004; cobaltous chloride, 0.0009; zinc carbonate, 0.0004; and sodium fluoride, 0.0001 (obtained from General Biochemicals, Inc., Chagrin Falls, Ohio).

The effect of cold exposure on retinoic acid requirement was assessed in experiment 4. Vitamin A-deficient rats were divided into 9 groups. Six groups were held at 5° and three were maintained at 25°. The rats at 5° were injected daily intraperitoneally with 2.5, 5, 10, 50 or 100 μg of retinoic acid in 0.1 M phosphate solution (pH adjusted to 10.2), and rats at 25° were injected daily with 2.5 or 5 μg of retinoic acid. The injection of retinoic acid was continued for 30 days or until death, whichever occurred sooner. Two groups of animals that were not given retinoic acid served as controls at each environmental temperature.

Analysis. All animals in experiments 1, 2 and 3 were decapitated. The livers were removed, dried by grinding with Na_2SO_4 and extracted 3 times with petroleum ether. The petroleum ether extract was evaporated to dryness under vacuum and made up to volume in cyclohexane for the determination of vitamin A ester by the 3-point correction method of Cama et al. (10) and for a gravimetric determination of total lipid.

Retinyl acetate supplement. Pure all-*trans* retinyl acetate [3] was dissolved in a solution of petroleum ether-acetone (4:1, v/v). To this mixture, 30 ml of cottonseed oil [4] and 150 mg of α-tocopheryl acetate [5] were added and mixed thoroughly. The solvent was evaporated under vacuum in a Rinco rotary evaporator. The concentration of retinyl acetate was determined spectrophotometrically. The two oil solutions prepared contained in 0.1 ml, 252 μg and 363 μg of retinyl acetate, respectively.

RESULTS AND DISCUSSION

The results of analysis for total vitamin A and total lipid in liver as well as the animal weight gains at 5° and 25° are presented in table 2. There was no sig-

[3] Distillation Products Industries, Rochester, New York.

[4] Wesson oil, Wesson Sales Company, Fullerton, California.

[5] Nutritional Biochemicals Corporation, Cleveland.

TABLE 2

Effect of environmental temperature on total vitamin A and lipid in liver and on weight gains of rats given retinyl acetate orally

Exp. no.	Days after supplementation	Temperature	No. of rats	Total liver lipid	Total vitamin A/liver	Total wt gain
				% liver wt	*μg*	*g*
1 [1]	0	25°	4	2.32 ± 0.11 [2]	1210 ± 75	—
	14	25°	6	2.29 ± 0.12	992 ± 82	38.2 ± 3.3
		5°	4	2.15 ± 0.14	894 ± 152	−2 ± 14.4 [3]
	28	25°	6	2.35 ± 0.08	981 ± 54	68.3 ± 9.1
		5°	5	2.17 ± 0.10	921 ± 34	19.8 ± 3.8 [3]
2 [4]	0	25°	4	1.90 ± 0.10	1350 ± 46	—
	42	25°	6	2.60 ± 0.24	1009 ± 21	140.3 ± 13.1
		5°	5	2.43 ± 0.30	1050 ± 21	80.2 ± 6.2 [3]
3 [4,5]	0	25°	4	2.18 ± 0.22	1427 ± 61	—
	28	25°	6	2.13 ± 0.14	1112 ± 56	88.8 ± 6.2
		5°	6	1.69 ± 0.07 [3]	1161 ± 68	60.8 ± 19.2

[1] 252 μg retinyl acetate administered daily for 9 days.
[2] SE of mean.
[3] Values with superscript 3 are significantly different from experimental animals at 25° at $P < 0.05$; Student's t test.
[4] 363 μg retinyl acetate administered daily for 6 days.
[5] Thiouracil administered at a level of 0.1% of diet from day 14 to 28.

nificant difference in total liver vitamin A levels or total lipids in livers of rats exposed to 5° for two, four or six weeks compared with their respective experimental controls maintained at 25°. However, the weight gains of rats at 5° were significantly less than those at 25° after two, four or six weeks of exposure to the environmental temperature (exps. 1 and 2, table 2; $P < 0.05$).

Johnson and Baumann (6) have reported that rats with a decreased growth rate (induced either by restricted caloric intake or by feeding diets low in thiamine or tryptophan) retained higher amounts of vitamin A in the liver than those that grew well. It was also shown that in rats of uniform size, the amounts of vitamin A retained varied inversely with the metabolic rate. Since exposure of rats to 5° causes a twofold increase in metabolic rate (11), rats exposed to 5° might then be expected to have a greater depletion of vitamin A than corresponding 25° controls. Although the total vitamin A levels in the liver of rats exposed to 5° did not differ significantly from those at 25° in our experiments (exps. 1 and 2), it is possible that the decreased rate of growth of rats in the cold, with its retarding effect on vitamin A utilization, might have counteracted the utilization of the vitamin promoted by the elevated metabolic rate.

We can possibly explain the effects of growth and cold exposure on the utilization of vitamin A if we adopt the method of Nir and Ascarelli (12) whereby the utilization of vitamin A is expressed as a ratio of vitamin A removed from the liver to weight gain (depletion ratio). Calculated in this manner, we obtain mean ratios of 3.6 ± 0.8 and 2.5 ± 0.2 (25° group) and 17.3 ± 5.0 and 3.8 ± 0.3 (5° group) after 4- and 6-week experimental periods, respectively. The differences between the mean depletion ratios of the 25° and 5° groups for the 4- and 6-week experimental periods were statistically significant ($P < 0.05$). It is not possible to calculate the mean depletion ratios for the 2-week exposed animals because of the negative weight change in the cold-exposed group. Although quantitative comparisons cannot be made, examination of the results shows qualitatively a large difference between the 25° and 5° groups in the 2-week exposed animals. Thus, we observe that there is an increase in vitamin A depletion from the liver per gram weight gain in the groups exposed to 5° for four and six weeks.

It is possible that vitamin A utilization is related to the increased thyroid activity during cold exposure, since it is known that the activity of the adrenals and thyroid gland increases during cold exposure

TABLE 3

Effect of graded amounts of retinoic acid on survival of rats at 5° and 25° (exp. 4)

Temperature	Amount injected daily	No. of rats	Initial body wt	30-day body wt gain [1]	No. of survivors
	μg		g		
5°	0	5	221.4 ± 15.6 [2]	—	0/5
	2.5	5	212.2 ± 7.4	—	0/5
	5.0	5	213.4 ± 6.3	—	0/5
	10.0	6	194.0 ± 11.5	—	1/6
	50.0	6	195.1 ± 9.2	—	0/6
	100.0	4	237.5 ± 19.4	14.0	2/4
25°	0	5	220.6 ± 6.4	−38.0	2/5
	2.5	5	212.0 ± 7.5	−60.0	3/5
	5.0	5	208.2 ± 21.7	25.5	4/5

[1] Of surviving animals.
[2] SE of mean.

(13, 14). To eliminate the effect of the increased thyroid activity during cold exposure, thiouracil was administered to both environmental temperature groups at a level of 0.1% of the vitamin A-free diet from day 14 to 28 of the experimental period. The results are presented in experiment 3 (table 2). The data show that the total liver vitamin A levels at 5° did not differ from those at 25°. Also, the weight gains in animals at 5° were not significantly different from those at 25°. The total liver lipids were significantly lower in the thiouracil-treated cold-exposed animals than in those at 25° ($P < 0.05$). When the mean depletion ratios are calculated, there is no difference between the 2 groups (3.8 ± 0.9 for 25° and 2.9 ± 1.2 for 5°). Thus, administration of thiouracil appears to have abolished the increase in the mean depletion ratio previously observed in the 4-week cold-exposed group (2.9 with thiouracil compared with 17.3 without thiouracil), though thiouracil does not alter the total vitamin A levels in the liver (exp. 3, table 2).

The results on the survival of vitamin A-deficient rats injected with different levels of retinoic acid are presented in table 3. It is evident that survival at 5° is markedly affected in rats deficient in vitamin A. Without retinoic acid supplementation, there were no survivors at 5°, and 2 rats still survived at 25° during the 30-day experimental period. At least 100 μg of retinoic acid was found necessary for survival and growth at 5° whereas approximately 5 μg of retinoic acid was sufficient for survival and growth at 25°. The 2 rats at 5° gained an average of 14 g in weight, and the 4 rats at 25° gained an average of 25.5 g in weight during the 30-day experimental period. Thus, it is clear from the foregoing experiment that the requirement for retinoic acid is markedly increased during cold exposure.

Ershoff (3) has shown a daily oral requirement for 0.9 μg of vitamin A for rats at room temperature and 1.7 μg for rats maintained in the cold. Because these requirements for vitamin A are so much less than those we found for retinoic acid, the results for the retinoic acid requirement may not be directly applicable to vitamin A which is the precursor of retinoic acid. Retinoic acid is not reduced to retinol and presumably not to retinal (7), but can be converted irreversibly to a metabolite with considerable biological activity (9). It may be that retinoic acid does not fulfill all the functions of vitamin A during acclimation to cold exposure, particularly with respect to the thyroid and the regulation of thyroxine secretion. Further study of the interrelationship between thyroid function and vitamin A is needed to afford a logical explanation.

Our results indicate that the requirement for retinoic acid and the utilization of vitamin A are increased during cold exposure when utilization of the vitamin is expressed as depletion of vitamin A from the liver per unit body weight gain. These observations are supported by those of Johnson and Baumann (6) and Nir and Ascarelli (12).

LITERATURE CITED

1. Phillips, W. E. J. 1962 Low-temperature environmental stress and the metabolism of vitamin A in the rat. Can. J. Biochem. Physiol., *40:* 491.
2. Ershoff, B. H. 1950 Effects of prolonged exposure to cold on the vitamin A requirement of the rat. Proc. Soc. Exp. Biol. Med., *74:* 586.
3. Ershoff, B. H. 1952 Effects of vitamin A malnutriture on resistance to stress. Proc. Soc. Exp. Biol. Med., *79:* 580.
4. Porter, E., and E. J. Masoro 1961 Effect of cold acclimation on vitamin A metabolism. Proc. Soc. Exp. Biol. Med., *108:* 609.
5. Johnson, R. M., and C. A. Baumann 1947 The effect of thyroid on the conversion of carotene into vitamin A. J. Biol. Chem., *171:* 513.
6. Johnson, R. M., and C. A. Baumann 1948 Relative significance of growth and metabolic rate upon the utilization of vitamin A by the rat. J. Nutr., *35:* 703.
7. Arens, J. F., and D. A. Van Dorp 1946 Biological activity of vitamin A acid. Nature, *158:* 622.
8. Dowling, J. E., and G. Wald 1960 The biological function of vitamin A acid. Proc. Nat. Acad. Sci., *46:* 587.
9. Yagishita, K., P. R. Sundaresan and G. Wolf 1964 A biologically active metabolite of vitamin A and vitamin A acid. Nature, 203: 410.
10. Cama, H. R., F. D. Collins and R. A. Morton 1951 Studies in vitamin A. 17. Spectroscopic properties of all-*trans* vitamin A and vitamin A acetate. Analysis of liver oils. Biochem. J., 50: 48.
11. Sellers, E. A., and S. S. You 1950 Role of the thyroid in metabolic responses to a cold environment. Amer. J. Physiol., *163:* 81.
12. Nir, I., and I. Ascarelli 1966 Effect of dietary protein level and thyroxine on vitamin A depletion from liver in chicks. Brit. J. Nutr., *20:* 41.
13. Sayers, G. 1950 The adrenal cortex and homeostasis. Physiol. Rev., *30:* 241.
14. Sellers, E. A. 1957 Adaptive and related phenomena in rats exposed to cold. Rev. Can. Biol., *16:* 175.
15. Lakshmanan, M. R., F. B. Jungalwala and H. R. Cama 1965 Metabolism and biological potency of 5,6-monoepoxy vitamin A aldehyde in the rat. Biochem. J., 95: 27.
16. Malathi, P., and J. Ganguly 1964 Studies on metabolism of vitamin A. 7. Lowered biosynthesis of ascorbic acid in vitamin A-deficient rats. Biochem. J., 92: 521.

Separation of Soybean Whey into Fractions with Different Biological Activities for Chicks and Rats [1]

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ABSTRACT Using a batch fractionation procedure on DEAE cellulose, 4 fractions of soybean whey were prepared and studied for biological activity with chicks and rats. All fractions prepared had trypsin inhibitor activity but hemagglutinin activity and intraperitoneal toxicity for rats was concentrated in one fraction. The fractions differed in relative stimulation of the pancreas of chicks to enlarge and the gallbladder to contract. Differences were also noted among the fractions in their effects on chick growth, fat absorption and dietary metabolizable energy value. Many of the effects caused by the soybean fractions appeared to be related to several components rather than to one specific fraction.

The influence of heat-labile factors present in defatted soybeans on animals consuming them has long been recognized and extensively studied. Mickelson and Yang (1) have most recently published an extensive review of work on this subject. Several investigators have shown that components of the soybean responsible for many of the effects in animals can be concentrated in the water-soluble, non-dialyzable portion, not precipitable at approximately pH 4 (2–4). This portion of the soybean, usually referred to as soybean whey, is known to consist of many separate components which possess such activity as inhibition of trypsin and chymotrypsin, hemagglutination, and several enzyme activities. Although several components with these in vitro activities have been isolated from the whey, their relationship to the physiological effects observed in animals fed the whey has not been extensively investigated. Separation of fractions of whey by chromatography on DEAE-cellulose columns has been accomplished by Rackis et al. (5) and by Stead et al. (6), who showed that components of the whey differed in their intraperitoneal toxicity to rats.

In recent studies in our laboratory, Garlich and Nesheim (3) were able to show that when soybean whey was fed at a low level (1.2%) to chicks, growth, fat absorption and metabolizable energy were depressed and the pancreas was enlarged, compared with similar measurements in chicks fed a diet containing heated, defatted soybeans. Two fractions of whey prepared by a batch fractionation method showed somewhat different biological activities. In addition, these fractions differed in trypsin inhibitor and hemagglutination activity.

The batch fractionation procedure appeared to be useful for preparation of quantities of whey fractions sufficient to fully evaluate the biological action of components of soybean whey. The present paper describes the developments of this procedure using DEAE-cellulose to prepare 4 fractions of soybean whey, which were tested for biological activity with chicks and rats. Measurements were made of several physiological effects known to be caused by defatted raw soybeans in an attempt to determine whether specific components of the whey were responsible for the various effects observed.

EXPERIMENTAL

Fractionation procedures. During several years of study of factors present in defatted soybeans, considerable variation in biological activity of various soybean preparations has been observed. This appar-

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ently was largely due to variations in heat treatment in defatting, removing solvent, or in drying the soybeans. Special care was taken, therefore, to select a good source of soybeans for these studies. Several different sources of beans were tested for their nitrogen solubility by the method of Lyman et al. (7), to detect any possible heat treatment that had been given to the soybeans. Soybeans of the Horasoy-63 variety purchased from the 1965 crop of an Illinois farm shortly after harvest, were used. The finely ground soybeans were defatted by extraction of 4-kg batches for 24 hours with 60 to 70° petroleum ether in a large modified Soxhlet-type apparatus. The residual fat was about 0.5 to 0.8% of the meal. The condensed solvent was cooled to about 55° by a cooling coil before coming into contact with the soybeans. Soybean whey was prepared from these soybeans using a method similar to that developed by Gar-

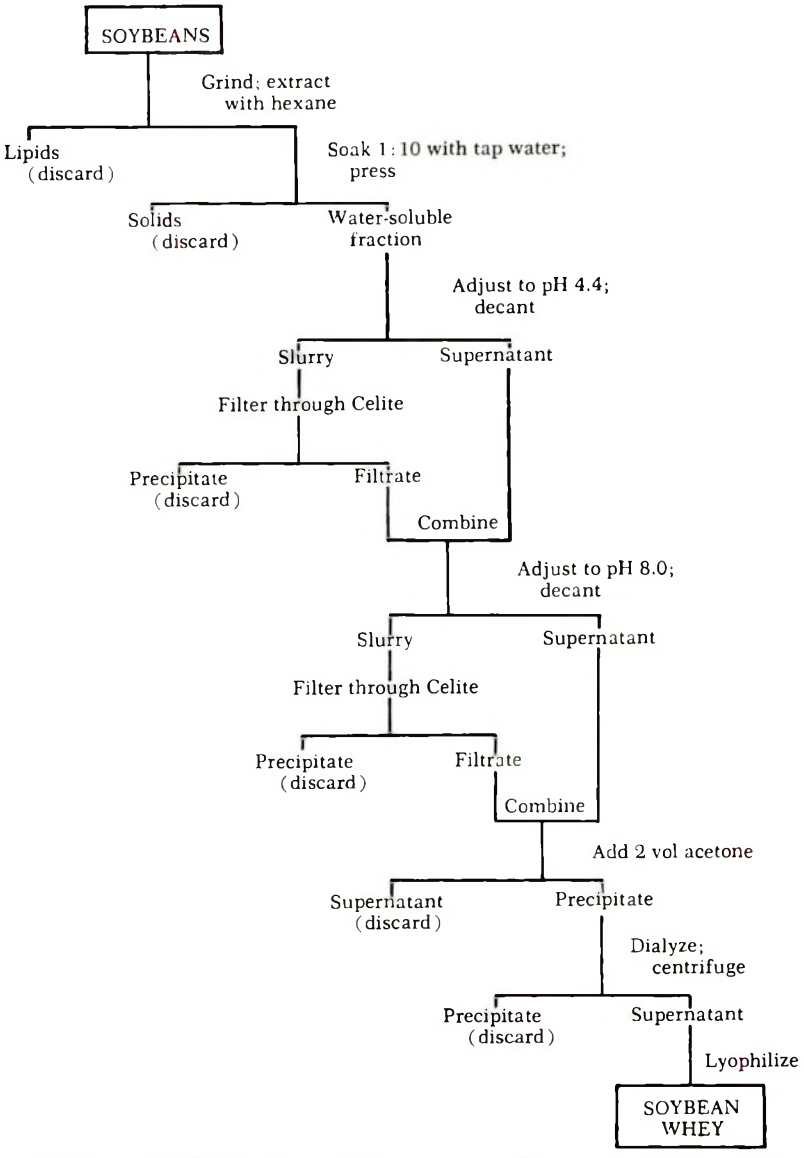

Fig. 1. Procedure followed to prepare dialyzed whey solids used for further fractionation.

lich and Nesheim (3). The outline of the whey preparation is shown in figure 1. Before dialysis, whey proteins were precipitated by acetone to reduce the quantity of material to be dialyzed. The whey solids recovered from this fractionation procedure represented about 1.2% of the original defatted soybeans.

The batch fractionation method of Garlich and Nesheim (2) was used. Three hundred grams of DEAE-cellulose were suspended in water and adjusted to pH 7.6 with saturated KH_2PO_4. The cellulose was poured onto a large Büchner funnel (32-cm diameter) and, with the aid of vacuum, washed with several liters of 0.01 M pH 7.6 phosphate buffer. Seventy-five grams of soybean whey were dissolved in 0.01 M pH 7.6 phosphate buffer and poured into the Büchner funnel. The packed funnel was washed with phosphate buffer until the protein content of the effluent (8) decreased to below 30% of the maximal value. The whole effluent containing protein was pooled and called fraction I. Fraction II was eluted from the Büchner funnel with 0.034 M NaCl in pH 7.6 0.01 M phosphate buffer. The first 2 liters of this effluent were discarded since the protein content was relatively low and appeared to be made up of components of fraction I. The Büchner funnel containing the cellulose and whey protein was washed with the eluent until the protein content of the effluent decreased to a low value. The funnel containing the remaining whey proteins on DEAE-cellulose was treated with the phosphate buffer containing 0.13 and 0.17 M NaCl in a manner similar to that used to obtain fractions III and IV. An attempt was made to recover a fifth fraction by using 0.25 M NaCl in buffer as a further wash, but this eluted only a small amount of additional material that had a chromatographic behavior similar to fraction IV.

A summary of the batch fractionation of the soybean whey is shown in table 1. The effluents making up each fraction were dialyzed against frequent changes of distilled H_2O at 4° until no chloride ion could be detected in the dialysis water. This usually required 48 hours. After dialysis the effluents making up the 4 fractions were centrifuged and the supernatants frozen and lyophilized. To obtain quantities of protein sufficient for the experiments to be described, the whole procedure had to be repeated 3 times. The whey fractions prepared in this manner were chromatographed on a 1-cm × 17-cm DEAE-cellulose column with a gradient elution procedure similar to that used by Rackis et al. (9). The protein content of the effluent was measured with an autoanalyzer.[3] The elution patterns of the 4 fractions (fig. 2) show chromatographic behavior of these fractions under identical conditions.

TABLE 1

Buffer sequence for elution of soybean whey fractions from Büchner funnel packed with DEAE cellulose

	Eluent	Effluent
1	Phosphate buffer pH 7.6, 0.01 M	fraction I
2	As (*1*) + 0.034 M NaCl	fraction II
3	As (*1*) + 0.13 M NaCl	fraction III
4	As (*1*) + 0.17 M NaCl	fraction IV

For heat-inactivated controls, defatted soybeans were autoclaved at 107° for 40 minutes. Before the heat treatment the soybeans were adjusted to 20% moisture and allowed to stand overnight at 4°. The autoclaved defatted soybeans were dried at 60° to the same water content as the unheated defatted soybeans used in these experiments.

Analytical procedures. Fat absorption was determined by the procedure of Renner and Hill (10) except that fecal fat was determined by extracting the dried excreta with ethyl ether for 12 hours in a Goldfisch apparatus. Preliminary investigation had indicated that no fecal soaps were present in the dried excreta. The metabolizable energy value of the experimental diets was determined according to the procedures of Hill and Anderson (11) except that the chromium in the diet and feces was determined by a slight modification of the method of Czernocki et al. (12).

Trypsin inhibitor activity in the soybean fractions was determined by measuring the inhibition of hydrolysis of N-benzoyl-DL-arginine *p*-nitroanilide by trypsin. The conditions for assay of trypsin were those used by Erlanger et al. (13). To assay trypsin inhibitor activity in the soybean frac-

[3] Technicon Chromatography Corporation, Chauncey, New York.

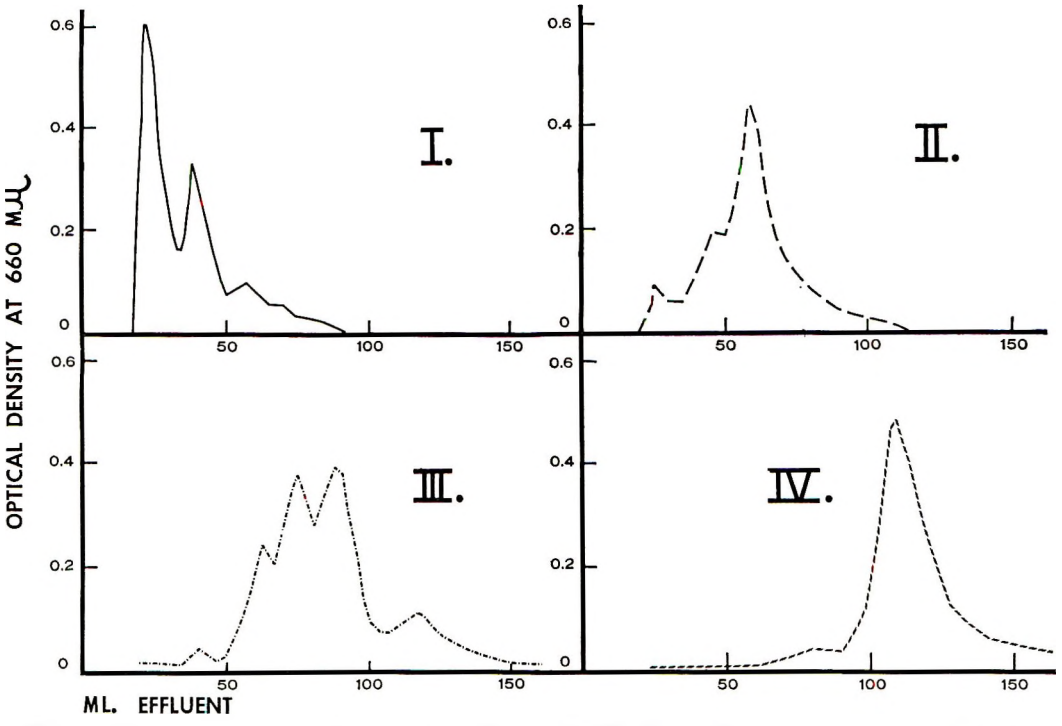

Fig. 2. Chromatographic patterns of fractions I to IV. Ten milligrams of each fraction chromatographed on 1 × 17 cm column of DEAE cellulose. Fractions were dissolved in 0.01 M pH 7.6 phosphate buffer and column was developed by gradient elution from 0 to 0.3 M NaCl in 0.01 M pH buffer. A mixing chamber of 160 ml was used. Protein in effluent measured by Lowry (8) reaction.

tions and soybean meal the material was either dissolved in, or extracted with, 0.0025 N HCl and mixed with an appropriate trypsin concentration before the trypsin assay. Trysin inhibitor activity was expressed in units related to the release of *p*-nitroaniline into the reaction mixture by the action of trypsin. Thus one trypsin unit was arbitrarily defined as the amount of trypsin that would cause an increase in optical density of substrate solution at 410 mμ in 10 minutes at 30° equivalent to that obtained from 0.01 μmoles of *p*-nitroaniline per 7 ml final volume of the reaction mixture. One trypsin inhibitor unit is the amount of inhibitor that would inhibit one trypsin unit. This method of expression of trypsin inhibitor units has the advantage that it is independent of the purity of the trypsin source used, and thus constant.

Hemagglutinin activity in the preparations studied was determined by the photometric procedure of Liener (14).

Chick experiments. Even though the fractionation technique used made it possible to prepare several grams of each of the fractions, a feeding experiment of short duration was necessary because of the small amounts of each fraction available. Several preliminary experiments were conducted to determine the age of chicks in which the effects of the defatted soybean meal were most pronounced, and also to ascertain the necessary length of the experimental period. These experiments showed that a 4-day experimental period beginning at the eighth day of age would adequately differentiate the effects of the fractions of soybean meal. Based on these observations, an experiment was designed to test the biological activities of the soybean fractions prepared. The diet used in this study, shown in table 2, is similar to that used previously in this laboratory. The basal diets containing either unheated defatted soybeans or autoclaved defatted soybeans served as negative and

TABLE 2
Basal diets used in chick experiments

	%
Raw or autoclaved defatted soybeans	50.00
Soybean oil	15.00
Glycine	0.50
Methionine	0.30
Chromic oxide premix (30% Cr_2O_3)	1.00
Mineral premix MCN-2 [1]	5.63
Vitamin premix MCN-1 [1]	1.25
Glucose monohydrate	26.32

[1] For composition see Nesheim et al. (23).

positive control treatments. The soybean fractions were all tested in the diet which contained heated soybean meal, and were added at the expense of an equal weight of glucose.

The soybean fractions were included in the diet on the basis of the estimated percentage each fraction represented of the original whey. This was estimated from a DEAE-cellulose chromatogram of the unfractionated whey. Thus, when a plot of the protein elution pattern from soybean whey was divided into areas with chromatographic behavior similar to each of the individual fractions, the total area was estimated to be 41% fraction I, 13% fraction II, 27% fraction III and 19% fraction IV. The actual levels fed were those corresponding to the amounts of each fraction expected to be present in 2% soybean whey adjusted for differences in nitrogen content. A commercially purchased trypsin inhibitor preparation was included in the diet at the same nitrogen level as that of fraction IV. This fraction had a similar chromatographic behavior on DEAE-cellulose as the purchased inhibitor. This inhibitor was apparently the same as isolated by Kunitz (15) and also called the A_2 inhibitor by Rackis et al. (9), based on its chromatographic behavior. In addition to feeding the soybean fractions separately, another treatment consisted of a combination of fraction I and the purchased soybean inhibitor to ascertain whether these 2 components of unheated soybeans might have a synergistic effect.

For the chick experiment, 250 Rhode Island Red × Barred Plymouth Rock male chicks purchased from a commercial hatchery were fed the basal diet containing the autoclaved defatted soybeans for 7 days. The feeders were removed each night to train the chicks to start eating at a desired time in the morning. This was necessary to facilitate measurements of gallbladder weight and intestinal nitrogen levels at the end of the experimental period.

On the eighth day the chicks were weighed and divided into 27 groups of 7 chicks each. Each group of chicks was made up to minimize the variation within the group, and chicks with either high or low extreme weights were discarded. Three groups of seven were then assigned at random to each of the 9 treatments and distributed to pens in 2 battery brooders after recording individual weights. The chicks were fed the experimental diets for 4 days. During the last 3 days of this period, excreta from each pen were collected and pooled. On the thirteenth day the experiment was concluded.

The following procedure was used at the completion of the experiment. Individual weight was determined and the birds were killed by dislocation of the neck. Following this, the pancreas and gallbladder (contents intact) were weighed in the fresh state, and the contents of the lower small intestine (not including duodenum) were forced out of the intestine by rolling a glass rod over the stretched intestine. The contents of the intestines from each chick in a pen were pooled, homogenized with distilled water and lyophilized.

Rat experiments. To measure the intraperitoneal toxicity of the soybean fractions, female rats of the Wistar strain, were fed a diet containing heated soybean meal supplemented with DL-methionine from weaning to an average of 52 g. The various fractions dissolved in 0.9% NaCl solution were administered intraperitoneally at various levels, to 10 rats per level of injection. The number of dead rats per 72 hours was used for a graphical determination of the LD_{50} by the procedure of Miller and Tainter (16).

In the studies of the $^{14}CO_2$ production after ingestion of DL-methionine-2-^{14}C, male Wistar rats were fed a stock diet from weaning to an average of 105 g. At the end of this rearing period the rats were fasted overnight (12 hours) and were then given by stomach tube 50 mg of each soybean fraction dissolved in water.

After one hour the rats were given 1 ml of a solution containing approximately 10 μCi DL-methionine-2-^{14}C by stomach tube, and were immediately placed in glass metabolism cages.[4] Expired air was trapped in 0.1 N NaOH. The NaOH in the absorption towers was changed hourly for the next 4 hours. Preliminary experiments showed that 4 hours was sufficient to differentiate the treatments. The experiment was repeated with 3 rats per fraction studied. The ^{14}C activity in the NaOH was counted in Bray's (17) solution in a liquid scintillation counter.

RESULTS

The procedures used to fractionate soybean whey resulted in the preparation of 4 fractions. The DEAE-cellulose chromatography of these 4 fractions is shown in figure 2. The fractionation procedure clearly produced 4 separate fractions differing in their chromatographic behavior on DEAE-cellulose. The resolution between the 4 fractions was not as good as could be anticipated by the usual chromatographic techniques. Thus there is considerable overlapping in the chromatographic pattern between fractions I and II and fractions II and III. However, fractions I, III and IV had relatively little material with similar chromatographic behavior. There was an appreciable amount of carbohydrate present in each fraction. This may have been due to the presence of carbohydrates in the protein or due to some DEAE-cellulose contamination in the preparation.

The results of studies with rats on the intraperitoneal toxicity of the fractions and effects on ^{14}C methionine oxidation are shown in table 3. Trypsin inhibitor and hemagglutinin activity of the individual fractions are also shown in this table. All fractions prepared had considerable trypsin inhibitor activity. The most active fraction was fraction III and the least active, fraction I. The hemagglutinin activity of the whey fractions was concentrated primarily in fraction I which had over 8 times the activity per unit of sample as fraction II. The other fractions had essentially no hemagglutinin activity.

When these fractions were injected into young rats, fraction I had the highest intraperitoneal toxicity. Fraction II had some activity but no toxicity was observed at the levels of injection used for fractions III and IV. Some toxicity of the soybean whey may have been lost during the fractionation procedure since fraction I and whey had the same LD_{50} when injected into rats. The fractions studied also differed in their ability to stimulate $^{14}CO_2$ production following a dose of DL-methionine-2-^{14}C. Rats dosed with fraction I had the lowest $^{14}CO_2$ recovery, whereas those given fraction III had the largest recovery of ^{14}C from methionine in the expired CO_2.

The results of the measurements made in an experiment with chicks are shown by the data in table 4. The fractions of the soybean whey had considerably dif-

[4] Delmar Scientific Laboratories, Chicago.

TABLE 3

Trypsin inhibitor and hemagglutinin activity, intraperitoneal toxicity and effects on ^{14}C-methionine oxidation by soybean fractions

Sample tested	Trypsin inhibitor activity	$^{14}CO_2$ recovery [1]	Intraperitoneal toxicity LD_{50} rats	Hemagglutinin activity
	units/mg air-dried sample		*mg/100 g body wt*	*units/mg air-dried sample*
Raw soybeans	39.6	—	—	26
Heated soybeans	0.0	—	—	0
Whey	1283	7.5 [bc]	90	496
Fraction I	1163	6.4 [c]	90	1321
Fraction II	1317	7.3 [bc]	350	160
Fraction III	2580	9.2 [a]	over 2000	47
Fraction IV	1405	8.0 [b]	over 2000	9
Trypsin inhibitor, lot 8822 [3]	1685	7.8 [bc]	over 2000	1

[1] In rats, following dose of DL-methionine-2-^{14}C; see text.
[2] Values not followed by same letter are significantly different by analysis of variance followed by Duncan's multiple range test (34). Three rats studied per fraction.
[3] Nutritional Biochemicals Corporation, Cleveland.

TABLE 4

Influence of fractions of soybean whey in a diet for chicks [1]

Exp. no.	Tested	Proportion of diet	Final body wt	Feed intake	Trypsin inhibitor units ingested	Pancreas wt adjusted [3]	Gall-bladder wt adjusted [3]	Fat absorption	ME value of diet, adjusted [4] (dry matter basis)		Nitrogen digestibility [5]
	No. observations/treatment		21	3	3	21	18	3	3	3	3
		%	*g*	*g*	*units* × 10^5	*mg*	*mg*	%	*kcal/g*	*kcal/g*	%
1	Raw soybeans	50	109 [a 6]	71	14.2	920 [a 7]	89 [a 7]	34 [a 7]	2.501	3.520 [a 7]	46 [ab 7]
2	Heated soybeans	50	134 [c]	70	0.0	527 [c]	167 [c]	87 [d]	3.570	3.778 [bc]	53 [abc]
3	Whey	2.00	124 [b]	83	21.3	895 [a]	112 [a]	52 [ab]	2.721	3.469 [a]	41 [a]
4	Fraction I	0.88	135 [c]	77	7.9	674 [b]	141 [bc]	79 [cd]	3.411	3.737 [bc]	63 [c]
5	Fraction II	0.32	132 [c]	75	3.2	648 [bc]	152 [c]	81 [cd]	3.510	3.806 [c]	62 [c]
6	Fraction III	0.56	128 [bc]	77	11.1	884 [a]	98 [a]	71 [bcd]	3.170	3.612 [ab]	44 [a]
7	Fraction IV	0.47	128 [bc]	78	6.2	850 [a]	83 [a]	64 [bc]	3.077	3.627 [b]	46 [ab]
8	Trypsin inhibitor (Kunitz)	0.35	133 [c]	80	4.7	827 [a]	99 [a]	71 [bcd]	3.176	3.618 [ab]	59 [bc]
9	Fraction I + trypsin inhibitor	0.88 0.35	124 [b]	74	12.0	931 [a]	112 [ab]	58 [b]	2.824	3.470 [a]	44 [a]

[1] Where 3 observations are indicated, the data were obtained as a pen average, otherwise number refers to observations taken on individual chicks.

[2] Experimental period lasted from day 8 to day 13 following hatching.

[3] Adjusted by analysis of covariance to a common body weight.

[4] Adjusted to 100% fat absorption. Fecal fat per gram of diet was considered to have gross energy of 9.4 kcal/g. The energy of the fecal fat was added to the determined metabolizable energy (ME) value to adjust for differences in fat absorption.

[5] Determined from analysis of intestinal contents.

[6] Values not followed by same letter differ significantly according to analysis of variance and Duncan's multiple range test, $P < 0.01$.

[7] As in footnote 6, but $P < 0.05$.

ferent biological activities in this experiment. All the fractions studied were included in a diet for chicks at very low levels compared with the original raw soybeans from which they were isolated. Since they were present in approximately the same amounts as they were found in intact whey, the contributions of these fractions to the total effect of the whey could be assessed.

Final weight was significantly depressed by feeding raw soybeans and the soybean whey as well as a combination of fraction I plus the Kunitz trypsin inhibitor. The other fractions studied did not significantly depress growth rate when fed by themselves. Feed intake was good in all treatments and none of the fractions appeared to have any marked effect upon feed consumption. Pancreas weight was increased by all samples except fraction II, but both fractions I and II had significantly less effect on the pancreas than the soybean whey, fractions III, IV, and the purchased trypsin inhibitor.

A recent observation in our laboratory has been that soybean fractions appear to cause a contraction of the gallbladder.[5] Young chicks (less than 2 weeks old) fed raw defatted soybeans continuously have a gallbladder that is nearly always empty of bile, whereas chicks fed heated defatted soybeans under the same conditions usually have considerable amounts of bile in the gallbladder. This phenomenon is illustrated by the data for gallbladder weight shown in table 3. Chicks receiving raw defatted soybeans had a gallbladder weight significantly less than those receiving heated soybeans. Chicks receiving the whey, fractions III and IV and purchased trypsin inhibitor had a gallbladder weight significantly less than those receiving heated defatted soybeans or fractions I and II.

The absorption of fat was markedly depressed in chicks receiving the diet containing the raw defatted soybeans compared with those receiving the heated materials. Fat absorption was also depressed in the chicks receiving the soybean whey and fraction IV as well as in the treatment with the combination of fraction I and the trypsin inhibitor. All treatments containing soybean fractions had numerically lower fat absorption values than those receiving the heated control treatment. In particular, fractions III and IV appeared to have a greater effect on fat absorption than fractions I and II, but due to the variation encountered in the fat absorption measurements, these differences were not statistically significant.

The metabolizable energy value of the diet was very low for chicks receiving the unheated defatted soybean treatment compared with the chicks receiving the heated control treatment. A difference of nearly one kilocalorie per gram of diet in the unadjusted metabolizable energy values was observed between these 2 diets. These differences are largely a reflection of the poor absorption of fat. It is possible to correct the metabolizable energy value of a diet to a 100% fat absorption basis by assuming that fecal fat contains 9.4 kcal of gross energy per g, and adjusting the metabolizable energy value obtained for the caloric value of the unabsorbed fat. By assuming that the digestibility of the fat does not affect the metabolizable energy value of the other compounds of the diet, the various treatments for the utilization of factors other than fat in the diets can be compared. Previous studies from this laboratory (18) indicated that where fat absorption is not affected, digestion of protein appears to be the strongest reason for differences in metabolizable energy value of diets containing unheated or heated defatted soybeans for chickens. Therefore, the differences in metabolizable energy value of the diets adjusted to 100% fat absorption are probably a reflection of differences in protein digestion. If this assumption is made, the chicks receiving the unheated defatted soybeans, whey, and fraction I plus the purchased trypsin inhibitor digested protein to a significantly less extent than those receiving the other treatments, based on the differences in adjusted metabolizable energy value of the diets.

The disappearance of intestinal nitrogen was determined by measuring the nitrogen and chromium content of the intestinal contents and the diets. Thus a loss of nitrogen in relation to chromic ox-

[5] Unpublished data, J. A. Serafin, Cornell University.

ide from the diet to the intestinal contents could be used as a measure of nitrogen digestibility at this stage of passage through the tract. The chicks that received raw soybeans, soybean whey, fraction III, IV or the combination of fraction I and the trypsin inhibitor, had the lowest disappearance of intestinal nitrogen or the lowest nitrogen digestibility. In general the treatments with a low adjusted metabolizable energy value also had the lowest values for nitrogen digestibility as measured in intestinal contents.

DISCUSSION

The batch fractionation procedure used for isolating components of soybean whey was reasonably successful. Although the fractions produced were not completely separated, this procedure does appear to be a reasonable method for fractionating soybean whey into components that can be studied for biological activity in feeding experiments. Conventional column chromatographic procedures are not suited for the isolation of the large quantities of material needed to do an adequate feeding experiment. The fractions produced by the batch technique used here are not pure single components but undoubtedly contain many separate components. These fractions would be useful starting material for further fractionation procedures using more refined techniques.

The soybean whey fractions isolated had considerably different biological activities. The intraperitoneal toxicity to rats was concentrated in fraction I. Although the hemagglutinin activity was also concentrated in this fraction, the compounds responsible for these activities may not be the same. Jaffe (19) assumed that toxic and hemagglutinin properties of *Phaseolus vlugaris* could be ascribed to the same components. However, Stead et al. (6) recently reported that chromatographically separate components of *P. vulgaris* were responsible for hemagglutinin activity and the intraperitoneal toxicity for rats. These authors reported that a fraction responsible for intraperitoneal toxicity of the soybeans was eluted from a DEAE-cellulose column at similar salt concentration as our fraction I. Factors responsible for hemagglutinin activity and intraperitoneal toxicity were not separated by our procedures.

All fractions studied had considerable trypsin inhibitor activity. Based on chromatographic behavior, fraction IV was made up primarily of the inhibitor first characterized by Kunitz (15) and later called the A_2 inhibitor by Rackis et al. (5). The identification of the components with trypsin inhibitor activity in the other fractions was not attempted. Rackis and Anderson (20) have described 4 trypsin inhibitors in soybean whey.

Although the fractions with the highest trypsin inhibitor activity per unit weight (III and IV) also had the greatest effect on the pancreas and gallbladder, the effects on these organs could not be completely correlated with the trypsin inhibitor activity. Thus chicks receiving fraction I consumed nearly twice as many inhibitor units as chicks receiving the Kunitz inhibitor, yet fraction I had relatively little influence on the gallbladder. Although chicks receiving fraction I did have a larger pancreas than chicks receiving the heated control diet, the stimulation was relatively small compared with chicks receiving fractions III and IV. Trypsin inhibitors from a wide variety of sources appear to cause pancreas stimulation but their overall biological effects may be quite different (3). The effect on ^{14}C-methionine oxidation appeared to be in proportion to trypsin inhibitor content of the fractions, in agreement with the results of Kwong and Barnes (21).

The data reported for gallbladder weights illustrate an effect of defatted soybeans that has not been reported previously. The influence on gallbladder weight appears to be primarily due to differences in weight of bile stored in the gallbladder. This effect can be readily demonstrated in chicks receiving unheated defatted soybeans if they are fed the diets continuously. If feed is removed two or three hours before autopsy, the gallbladders of chicks receiving unheated defatted soybeans are usually not greatly different from those receiving the heat-inactivated control diet. The effect of the soybeans or soybean fractions appears to be one of causing the gallbladder to contract and expel the bile. Fractions that are most stimulatory to the

pancreas also appear to be the most stimulatory to the gallbladder. The purified trypsin inhibitor was quite effective in stimulating the gallbladder to contract. It is of interest that Jorpes and Mutt (22) have recently published evidence that the gastrointestinal hormones, pancreazymin and cholecystokinin are the same compound. Thus if the pancreas stimulation was mediated by release of a gastrointestinal hormone such as pancreozymin, the effect on the gallbladder observed in this experiment would be expected.

The effects of the treatments on fat absorption were not clear-cut. In confirmation of previous studies (23, 24), the unheated defatted soybeans markedly depressed fat absorption. Chicks consuming the fractions which caused the gallbladder to contract also had relatively poor fat absorption. Chicks receiving defatted soybeans apparently are deficient in bile acids (25). The significance of the influence of the soybean fractions on the gallbladder and the defect in fat absorption remain to be determined.

The fractions with the greatest effect on metabolizable energy content of the fat-free portion of the diet appear to be the defatted raw soybeans, soybean whey and fractions III, IV or the combination of fraction I plus the Kunitz inhibitor. From previous studies we would expect the low metabolizable energy value of these treatments to be a reflection of poor overall protein digestibility. Thus it appears that some trypsin inhibitors might be more effective than others in inhibiting protein digestion.

Several investigators (4, 26, 27) have suggested that trypsin inhibitors cause a stimulation of the pancreatic secretions which results in the loss from the body of a large number of essential amino acids required for growth of the animal. Recently Khayambashi and Lyman (28), with an amino acid diet and a trypsin inhibitor concentrate, demonstrated apparently large losses of essential amino acids in protein which must have arisen from the intestinal tract, possibly through the activity of the pancreas. These studies show that substantial losses of endogenous protein must occur. It appears, however, that another important feature of this argument must be that these losses of protein, endogenous or dietary, occur because of inhibition of proteolytic activity in the intestinal tract by the trypsin and chymotrypsin inhibitors present in the soybeans. Otherwise there is no good reason to expect that the endogenous secretions would not be digested and reabsorbed. Protein digestibility by chicks receiving unheated defatted soybeans has been shown by a number of workers to be quite low (18, 29, 30). Alumot and Nitsan (31) demonstrated that proteolytic activity in the intestinal tract is markedly depressed in chicks by feeding unheated soybeans, However, proteolytic activity usually has been shown to be elevated in the intestinal tract of rats receiving unheated soybean meal (26). But most of these measurements have been of total proteolytic activity with a protein substrate and not of the activity of specific digestive enzymes such as trypsin or chymotrypsin. If the activity of these important endopeptidases was inhibited despite a large amount of apparent proteolytic activity toward a protein substrate, large fragments of protein in the intestine may not be broken down because of the inhibition of these endopeptidases. Protein digestibility in rats receiving unheated soybean meal has been reported to be depressed much less than in chicks. Because of the microbiological activity in the lower digestive tract of rats, unabsorbed nitrogen may be released as ammonia and be reabsorbed. This would not be measured in a conventional nitrogen digestibility study. This possibility has been pointed out previously by Carroll et al. (32) and demonstrated by Nesheim and Carpenter (33) to take place in chicks receiving poorly digested proteins.

In general the fractionation studies reported here indicate that the effects of soybean whey on growth rate, pancreas enlargement, gallbladder contraction, fat absorption and metabolizable energy value of the diet cannot necessarily be ascribed to a single component of the soybean whey. Activity for these various effects appeared to be distributed among the 4 fractions studied, although some appeared to be much more active than others toward a specific effect. It is conceivable that combinations of the various components of soybean whey actually act in a synergis-

tic fashion. Thus the effects of fraction I plus the Kunitz trypsin inhibitor (treatment 9, table 3) appear to be considerably greater than the summation of the effects of the individual fractions. Although this may be only an expression of additive effects, it is conceivable that the trypsin inhibitor protected the components of fraction I from digestion so that they could exert more of an effect. Certainly if the complex actions of the components of soybean whey on the animal are to be sorted out, one must consider the possibility that interactions of more than one component may cause the final result obtained when the animal is fed the legume fractions.

It appears likely that soybean whey is made up of several components, most likely proteins which may have the ability to have a physiological influence on the intestinal tract of the animal. Thus it is unlikely that a specific pancreas hypertrophy factor, fat absorption-depressing factor or gallbladder-stimulating factor can be isolated. The general properties of several proteins—solubility, molecular weight, action against trypsin and chymotrypsin, net charge at physiological pH—may be responsible for the biological influence they have. For example, although a crystalline soybean trypsin inhibitor has been shown to cause pancreatic hypertrophy, several other trypsin inhibitors probably will do the same thing (3).

Other effects of soybeans, particularly the intraperitoneal toxicity for rats, may well be caused by a specific component that can be isolated with present techniques.

LITERATURE CITED

1. Mickelson, O., and M. G. Yang 1966 Naturally occurring toxicants in foods. Federation Proc., *25:* 104.
2. Rackis, J. J., A. K. Smith, A. M. Nash, D. J. Robbins and A. N. Booth 1963 Feeding studies on soybeans. Growth and pancreatic hypertrophy in rats fed soybean meal fractions. Cereal Chem., *40:* 531.
3. Garlich, J. D., and M. C. Nesheim 1966 Relationship of fractions of soybeans and a crystalline soybean trypsin inhibitor to the effects of feeding unheated soybean meal to chicks. J. Nutr., *88:* 100.
4. deMuelenaere, H. J. H. 1964 Studies on the digestion of soybeans. J. Nutr., *82:* 197.
5. Rackis, J. J., H. A. Sasame, R. L. Anderson and A. K. Smith 1959 Chromatography of soybean proteins. I. Fractionation of whey proteins on diethyl-aminoethyl-cellulose. J. Amer. Chem. Soc., *81:* 6265.
6. Stead, R. H., H. J. H. deMuelenaere and G. V. Quicke 1966 Trypsin inhibition, hemagglutination, and intraperitoneal toxicity in extracts of *Phaseolus vulgaris* and *Glycine max.* Arch. Biochem. Biophys., *113:* 703.
7. Lyman, C. M., W. Y. Chang and J. R. Couch 1953 Evaluation of protein quality in cottonseed meals by chick growth and by a chemical index method. J. Nutr., *49:* 679.
8. Lowry, O. H., N. J. Rosebrough, A. R. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
9. Rackis, J. J., H. A. Sasame, R. L. Anderson, A. K. Smith and R. K. Mann 1962 Soybean trypsin inhibitor: Isolation, purification, and physical properties. Arch. Biochem. Biophys., *98:* 471.
10. Renner, R., and F. W. Hill 1960 Studies of the effect of heat treatment on the metabolizable energy value of soybeans and extracted soybean flakes for the chick. J. Nutr., *70:* 219.
11. Hill, F. W., and D. L. Anderson 1958 Comparison of metabolizable energy and productive energy determinations with growing chicks. J. Nutr., *64:* 587.
12. Czarnocki, U., I R. Sibbald and E. V. Evans 1961 The determination of chromic oxide in samples of feed and excreta by acid digestion and spectrophotometry. Can. J. Animal Sci., *41:* 167.
13. Erlanger, B. F., N. Kokowsky and W. Cohen 1961 The preparation and properties of two new chromatogenic substrates of trypsin. Arch. Biochem. Biophys., *95:* 271.
14. Liener, I. E. 1955 The photometric determination of the hemagglutinating activity of soyin and crude soybean extracts. Arch. Biochem. Biophys., *54:* 223.
15. Kunitz, M. 1945 Crystallization of a trypsin inhibitor from soybean. Science, *101:* 668.
16. Miller, L. C., and M. L. Tainter 1944 Estimation of the ED_{50} and its error by means of logarithmic probit graph paper. Proc. Soc. Exp. Biol. Med., *57:* 261.
17. Bray, G. A. 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem., *1:* 279.
18. Nesheim, M. C., and J. D. Garlich 1966 Digestibility of unheated soybean meal for laying hens. J. Nutr., *88:* 187.
19. Jaffé, W. G. 1960 Ueber Phytotoxine aus Bohnen (*Phaseolus vulgaris*) Arzneimittel-Forsch., *12:* 1012.
20. Rackis, J. J., and R. L. Anderson 1964 Isolation of four soybean trypsin inhibitors by DEAE-cellulose chromatography. Biochem. Biophys. Res. Commun., *15:* 230.
21. Kwong, E., and R. H. Barnes 1963 Effect of soybean trypsin inhibitor on methionine and cystine utilization. J. Nutr., *81:* 392.
22. Jorpes, E., and V. Mutt 1966 Cholecystokinin and pancreozymin, one single hormone? Acta Physiol. Scand., *66:* 196.

23. Nesheim, M. C., J. D. Garlich and D. T. Hopkins 1962 Studies on the effect of raw soybean meal on fat absorption in young chicks. J. Nutr., *78:* 89.
24. Brambila, S., M. C. Nesheim and F. W. Hill 1961 Effect of trypsin supplementation on the utilization by the chick of diets containing raw soybean oil meal. J. Nutr., *75:* 13.
25. Garlich, J. D., and M. C. Nesheim 1965 Effect of sodium taurocholate on fat malabsorption induced by feeding unheated soybean proteins. Proc. Soc. Exp. Biol. Med., *118:* 1022.
26. Haines, P. C., and R. L. Lyman 1961 Relationship of pancreatic enzyme secretion to growth inhibition in rats fed soybean trypsin inhibitor. J. Nutr., *74:* 445.
27. Barnes, R. H., and E. Kwong 1965 Effect of soybean trypsin inhibitor and penicillin on cystine biosynthesis in the pancreas and its transport as exocrine protein secretion in the intestinal tract of the rat. J. Nutr., *86:* 245.
28. Khayambashi, H., and R. L. Lyman 1966 Growth depression and pancreatic and intestinal changes in rats force-fed amino acid diets containing soybean trypsin inhibitor. J. Nutr., *89:* 455.
29. Lepkovsky, S., F. Furuta, T. Koike, N. Hasegawa, M. K. Dimick, K. Krause and F. J. Barnes 1965 The effect of raw soya beans upon the digestion of proteins and upon the function of the pancreas of intact chickens and of chickens with ileostomies. Brit. J. Nutr., *19:* 41.
30. Nitsan, Z. 1965 The effect of heating soybean meal on the apparent digestibility and metabolism of protein, methionine and lysine by cockerels. Poultry Sci., *44:* 1036.
31. Alumot, E., and Z. Nitsan 1961 The influence of soybean antitrypsin on the intestinal proteolysis of the chick. J. Nutr., *73:* 71.
32. 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.
33. Nesheim, M. C., and K. J. Carpenter 1967 The digestion of heat-damaged protein. Brit. J. Nutr., *21:* 399.
34. Duncan, D. B. 1955 Multiple range and multiple *F* tests. Biometrics, *11:* 1.

Metabolism of L-Arabinose and D-Xylose by Chicks [1]

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ABSTRACT Studies are reported on metabolism by chicks of dietary and parenteral (labeled) L-arabinose and D-xylose. Blood hematocrit, total reducing sugars, cholesterol, serine and proline in plasma and total reducing sugars in excreta increased due to graded (10, 20 and 40%) levels of dietary pentoses. Pentose treatments decreased liver weight, liver lipids and glutamic acid and lysine in plasma. Arabinose but not xylose decreased plasma alkaline phosphatase activity and potassium ion and increased chloride. The increases in total reducing sugars in plasma were found to be due to ingested pentoses. Radioisotope studies showed that L-arabinose is better metabolized than D-xylose by the chicks, but that neither pentose is metabolized to CO_2 as rapidly as D-glucose. The metabolic fate of 1-^{14}C label and possible pathways for these pentoses are discussed.

The inability of chicks to utilize L-arabinose and D-xylose at high dietary levels was reported in an earlier communication (1). These pentoses increased the plasma content of total reducing sugars and severely depleted liver and muscle glycogen.

This investigation was initiated to determine the influence of dietary L-arabinose and D-xylose on a variety of biochemical characteristics as well as the metabolic disposition of the labeled pentose sugars.

EXPERIMENTAL [3]

Male chicks (New Hampshire × Columbian) were used. Randomized complete block design in space (exp. 1) and in time (exp. 2) was used. Where pertinent, the treatment values were reported by Duncan's multiple range test (2) to detect significant differences among means.

Experiment 1. The composition of diets is shown in table 1. L-Arabinose and D-xylose were substituted at the expense of D-glucose on a moisture-free basis. Chicks reared in electrically heated battery brooders were fed a corn-soybean type diet, C5F (3) for 2 weeks after which they were fed the 60% D-glucose diet until 20 days of age. At termination of the study (24 days of age), plasma prepared from heparinized blood from individual birds in each pen was pooled and analyzed for the following components using a continuous analysis system[4,5]: calcium, phosphorus, cholesterol, total protein, albumin, uric acid, urea nitrogen, creatinine, sodium, potassium, chloride, alkaline phosphatase and total reducing sugars. Free amino acids in plasma (using picric acid deproteination) were determined by the method of Hamilton (4) using an amino acid analyzer.[6]

Deproteinized plasma samples were desalted by passing through strongly cationic (Dowex 50W-X4) and weakly anionic (Dowex 3-X4) exchangers. After concentrating the effluents, sugars were separated by descending paper chromatography using pyridine-ethyl acetate-water (2:5:7 v/v, upper phase) and detected by alkaline silver nitrate (5). Quantitation of D-glucose was accomplished by the glucose-oxidase method (6).

Dried and finely ground excreta samples were extracted twice in deionized water and centrifuged at 3600 rev/min for 15 minutes. The pooled supernatants

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[4] Technicon Corporation, Ardsley, New York.

[5] Technicon Laboratory (1963). Technicon Autoanalyzer Methodology, N-method files: N-3a for calcium, N-4b for phosphorus, N-24 for cholesterol, N-14b for total protein, N-15b for albumin, N-13b for uric acid, N-10a for urea nitrogen, N-11b for creatinine, N-21a for sodium and potassium, N-5a for chloride, N-28P for alkaline phosphatase and N-2a for total reducing sugars.

[6] See footnote 4.

TABLE 1
Composition of diets (exp. 1)

D-Glucose [1]	60
L-Arabinose [1]	(0, 10, 20, 40) [2]
D-Xylose [1]	(0, 10, 20, 40) [2]
Constant ingredients:	
Mineral mixture P-6 [3]	6
Vitamin mixture P-2 [4]	1
Vitamin mixture ADE-1 [5]	0.10
Other [6]	33.11

[1] Mann Research Laboratories, Inc., New York.
[2] Numbers in parentheses indicate levels of substitution for D-glucose.
[3] Mineral mixture P-6: (in grams) calcium phosphate (dibasic) dihydrate, 2.11; calcium carbonate, 1.69; potassium phosphate (monobasic), 1.12; sodium chloride, 0.65; magnesium sulfate (72%), 0.35; (in milligrams) ferrous sulfate heptahydrate, 33.3; manganese sulfate monohydrate, 33.3; potassium iodide, 0.65; cuprous sulfate pentahydrate, 1.67; zinc carbonate, 9.6; sodium molybdate dihydrate, 0.5; and sodium selenite, 21.8 μg.
[4] Vitamin mixture P-2, premix in 10.0 g dextrose·H_2O per kilogram diet: (in milligrams) biotin, 0.25; pyridoxine·HCl, 4; folic acid, 4; riboflavin, 10; thiamine·HCl, 10; vitamin B_{12} (0.1% triturate), 20; Ca D-pantothenate, 20; niacin, 50; and menadione sodium bisulfite (63%), 1.
[5] Vitamin mixture ADE-1, premix in 1 g corn oil: vitamin A palmitate, 10,000 IU; vitamin D_3, 1,000 ICU; and vitamin E (*d*-α-tocopheryl acetate), 27.2 IU.
[6] Other constant ingredients: (%) isolated soybean protein (Assay Protein C-1, Skidmore Enterprises, Cincinnati), 28.3; cellulose (Solka Floc, Brown Company, Gorham, New Hampshire), 2.0; DL-methionine, 0.4; glycine, 0.2; corn oil, 2.0; and choline chloride solution (70% w/w), 0.214.

were decolorized by activated carbon and analyzed for total reducing sugars as above.

Experiment 2. Unfasted chicks (16–22 days old) reared with diet C5F (3), were injected subcutaneously with 5 μCi of chromatographically and autoradiographically pure 1-^{14}C-labeled D-glucose [7] (1.35 μCi/μmole), L-arabinose [8] (0.45 μCi/μmole) and D-xylose [9] (0.62 μCi/μmole). Radioactivity in expired CO_2 in 6 hours was determined with the aid of an electrometer [10] based upon high-resistance-leak method (7). At the end of the experimental period, the liver glycogen was isolated from each chick (8). The radioactivity in glycogen and "water-soluble" and "insoluble" fractions of excreta were determined using liquid scintillation.

RESULTS

Experiment 1. Data pertaining to the performance and biochemical effects of dietary arabinose and xylose are presented in table 2. Increased dietary pentose levels decreased growth and efficiency of feed utilization. Both pentoses at the 40% level in the diet effected severe growth retardation and depression in feed efficiency.

Marked hemoconcentration was observed among birds receiving the highest dietary pentose levels. Inclusion of pentoses at intermediate levels tended toward increased hemoconcentration.

The two higher levels of arabinose in the chick's diet resulted in elevations in total reducing sugars in blood plasma, although these differences were not statistically significant. Proportional increments in total reducing sugars were observed due to increase in dietary level of xylose, confirming the earlier report (1). Paper chromatographic separations of plasma reducing sugars showed additional spots corresponding in position with standards and in intensity with amounts expected by calculating the differences between total reducing sugars and true glucose (see calculation in table 2) among the birds fed arabinose and xylose. The method was sensitive enough to detect the presence of arabinose in the plasma of chicks fed the 10% dietary level. The quantity of D-glucose in the plasma by glucose oxidase method indicated that D-glucose value remained fairly constant.

A consistent increase in the cholesterol content of the plasma was observed due to pentose treatment, corroborating the previous report (1). Alkaline phosphatase activity was significantly lower among birds receiving arabinose. As little as 10% arabinose in the diet lowered the enzyme activity to 60% of the control value. Ion concentration of potassium was lower and that of chloride higher in the plasma of chicks fed arabinose.

A few trends in the free amino acid content of blood plasma as influenced by dietary carbohydrates were observed. The serine level was higher and glutamic acid was lower in plasma of birds fed pentoses. The amount of proline in plasma was higher in birds receiving 40% level of arabinose and two higher levels of xylose. Arabinose at the two higher levels decreased the lysine concentration in plasma.

[7] Volk Radiochemical Company, Skokie, Illinois.
[8] Nuclear Research Chemicals, Inc., Orlando, Florida.
[9] See footnote 7.
[10] Cary Vibrating Reed Electrometer manufactured by Applied Physics Corporation, Monrovia, California.

TABLE 2

Effect of dietary levels of L-*arabinose and* D-*xylose on the performance and biochemical characteristics of chicks (exp. 1)* [1]

	Dietary carbohydrate, %						
	D-Glucose	L-Arabinose			D-Xylose		
	60	10	20	40	10	20	40
Weight gain, g	78.3 [a 2]	74.7 [ab]	67.7 [ab]	21.1 [c]	70.6 [ab]	63.1 [b]	24.0 [c]
Gain/feed	0.58 [a]	0.52 [a]	0.49 [a]	0.20 [b]	0.57 [a]	0.53 [a]	0.28 [b]
Hematocrit, %	24.7 [a]	26.8 [ab]	26.6 [ab]	28.1 [b]	25.3 [ab]	25.7 [ab]	27.9 [b]
Blood plasma (per 100 ml)							
Total reducing sugar, mg	343 [a]	325 [a]	440 [ab]	428 [ab]	473 [ab]	633 [b]	921 [c]
D-Glucose, mg [3]	341	324	368	345	336	353	356
L-Arabinose, mg [4]	0	1	72	83	0	0	0
D-Xylose, mg [4]	0	0	0	0	137	280	565
Cholesterol, mg	169 [a]	175 [ab]	204 [bcd]	211 [cd]	184 [abc]	218 [d]	196 [abcd]
Alkaline phosphatase, KA units [5]	643 [a]	386 [b]	292 [b]	290 [b]	685 [a]	692 [a]	587 [a]
Potassium, mEq	0.47 [abc]	0.36 [c]	0.39 [abc]	0.37 [bc]	0.48 [ab]	0.50 [a]	0.44 [abc]
Chloride, mEq	11.5 [a]	11.6 [ab]	12.3 [bc]	12.4 [c]	12.0 [abc]	12.0 [abc]	11.4 [a]
Amino acids							
Serine, μmoles	61 ± 10 [6]	92 ± 17	81 ± 24	110 ± 12	87 ± 9	97 ± 17	108 ± 23
Glutamic acid, μmoles	73 ± 18	23 ± 5	30 ± 9	31 ± 10	38 ± 9	33 ± 10	37 ± 8
Proline, μmoles	22 ± 1	32 ± 14	20 ± 1	48 ± 5	38 ± 7	60 ± 6	56 ± 15
Lysine, μmoles	93 ± 23	97 ± 2	68 ± 18	61 ± 5	64 ± 1	79 ± 5	80 ± 5
Liver							
Weight, g [7]	34.4 [a]	36.2 [a]	34.5 [ab]	30.9 [b]	37.6 [a]	35.9 [a]	33.9 [ab]
Total lipids, % (dry basis)	13.6 [a]	7.7 [b]	7.2 [b]	4.9 [b]	8.3 [b]	6.5 [b]	5.6 [b]
Excreta (dry basis)							
Total reducing sugar, mg/g	1.36 [a]	1.48 [ab]	2.46 [bc]	3.41 [c]	1.22 [a]	2.98 [c]	4.67 [d]
Total lipids, %	5.50 [a]	5.14 [a]	4.15 [ab]	3.67 [ab]	2.72 [b]	2.26 [b]	1.97 [b]

[1] Treatment values are averages of triplicate lots of 3 chicks each. Experimental period was from 21–24 days of age.

[2] Treatment values not followed by the same letter are significantly different at $P < 0.05$.

[3] Values determined by glucose oxidase method.

[4] Values for arabinose and xylose represent difference between total reducing sugars and D-glucose values. Zero values indicate the absence of pentoses by paper chromatography.

[5] King Armstrong units.

[6] Mean ± SD.

[7] Values reported on wet basis per kilogram body weight and are adjusted means after removal of body weight variation by covariance analysis.

TABLE 3

Metabolic disposition of radiolabeled pentoses in chicks (exp. 2) [1]

Compound	Recovered radioactivity					Retained
	$^{14}CO_2$	Liver glycogen [2]	Excreta [3]		Total	
			Soluble fraction	Insoluble fraction		
	%	%	%	%	%	%
D-Glucose-1-^{14}C	34.2	0.04 [a]	1.10 [a]	0.41 [a]	35.7	64.3
L-Arabinose 1 ^{14}C	4.57	0.04 [a]	13.5 [b]	4.39 [b]	22.5	77.5
D-Xylose-1-^{14}C	3.95	0.04 [a]	21.6 [b]	2.66 [b]	28.2	71.8

[1] Treatment values average of 3 chicks/treatment. Average weight of chicks was 244.3 ± 3.3 ($s_{\bar{x}}$) g. Randomized block design (in time) was used. Each chick was injected subcutaneously with 5 μCi of radiolabeled sugar.

[2] Recovery of radioactivity from liver glycogen is based on the assumption that the glycogen content of the liver was 16 mg/g of liver (1).

[3] The entire excreta from each bird was homogenized twice in 10 ml of distilled water, and centrifuged at 5,000 rev/min for 10 minutes. The supernatants were combined and concentrated to dryness. These were designated "soluble fraction." The sediment was dried at 65° for 8 hours and ground finely and designated "insoluble fraction."

[4] See table 2, footnote 2.

Total reducing sugars in excreta increased with graded levels of dietary pentoses. Excretion of reducing sugars among birds fed 40% xylose was 3.4 times higher than those fed the glucose diet. Total lipids in liver and excreta decreased with increased dietary intake of either pentose. Birds receiving xylose excreted very low amounts of fat.

Other components of plasma, viz., calcium, phosphorus, total protein, albumin, uric acid, urea nitrogen, creatinine, sodium, aspartic acid, threonine, glycine, alanine, valine, cystine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, histidine and arginine and also the bone ash content were not significantly influenced by dietary pentoses and hence data are omitted.

Experiment 2. Recovery and retention of radioactivity from radiolabeled sugars injected into chicks are shown in table 3. The amount of $^{14}CO_2$ expired by the chicks injected with labeled glucose in 6 hours was much higher than that of those receiving labeled pentoses.

The ^{14}C label was pooly incorporated into the liver glycogen from 1-^{14}C-labeled D-glucose, L-arabinose and D-xylose within 6 hours after administration. Although the recovery of ^{14}C in glycogen was so low (0.04%), the count rate for glycogen was consistently higher than the background.

Comparatively, little activity (1.5%) was observed in both "water-soluble" and "insoluble" fractions of excreta following radioactive glucose. Birds receiving labeled arabinose and xylose excreted 17.9% and 24.2%, respectively, of the administered activity. Only 35.7, 22.5 and 28.2% of the total radioactivity of glucose, arabinose and xylose, respectively, were recovered in expired CO_2, excreta and liver glycogen, indicating considerable retention.

DISCUSSION

Pronounced hemoconcentration has been reported in rats during depletion of 10% of the body weight, followed by hemodilution as depletion progressed up to 40% due to restricted protein intake (9). It is suggested that the observed hemoconcentration with higher levels of arabinose and xylose may be related to decreased weight gain. Decrease in plasma volume due to arabinose is also accompanied by reduction in alkaline phosphatase activity and change in ionic concentration (K^+ and Cl^-). Decrease in alkaline phosphatase activity may be due to direct interference by the pentose itself, to retarded enzyme synthesis, or to activity changes associated with pH of plasma. L-Arabinose may have utility as an inhibitor of alkaline phosphatase activity. Xylose was not inhibitory, nor did it influence K^+ and Cl^-. Decrease in plasma lysine among chicks receiving arabinose is an unexpected observation. Eckel et al. (10) observed that in rats a loss in muscle potassium is at least partially compensated for by a gain in free lysine which acts as a cation. In view of the previous work (1), decreased glutamic acid appears

to be an implication of reduced citric acid cycle activity due to energy deficiency or modified metabolic pathways.

The decrease in the amount of liver fat in relation to high intake of pentoses may reflect greater net utilization of body fat in general. The excretion of fat in these chicks was lower than in control birds. If the chicks were using depot fat to compensate for the lower available energy, this may well mean that net fat synthesis in these chicks is minimal.

Histochemical observations on liver showed that birds fed 40% arabinose suffered severe glycogen depletion as evidenced by lighter coloration with magenta stain as compared with control and xylose treatments, in agreement with the chemical data reported (1). Cytological examination of the livers stained with hematoxylin and eosin indicated that the sinusoids from birds fed arabinose were highly compressed as compared with those of the control birds. This may be due to the hydropic action on the liver cells. Although the cell nuclei appeared to be normal, vesicular spaces with no distinct cell boundaries were noticed. Birds receiving 40% of xylose indicated pronounced cytopyknosis of liver cells. The inability of liver cells to grow or divide at these levels was observed. Photomicrographic details of cytological studies are described.[11] These observations suggest that higher levels of dietary pentoses involve toxicity.

The appearance of higher amounts of xylose than arabinose in plasma may be due to the faster velocity of absorption and hence the net absorption of the former from the gastrointestinal tract (3) or higher kidney threshold, or both of these. Both xylose and glucose appear to be more efficiently reabsorbed than arabinose in agreement with intestinal absorption data (3). Wyngaarden et al. (11) observed increases in blood glucose levels and serum inorganic phosphate following infusions of D-xylose and L-arabinose in man. Present studies show that neither blood glucose nor plasma inorganic phosphate was affected in chicks due to dietary treatments.

It is believed that higher amounts of total reducing sugars in the excreta due to increased levels of dietary pentoses and the appreciable amount of radioactivity in excreta (table 3) of chicks injected subcutaneously are a reflection of pentose excretion via the urine. Due to experimental limitations urine and feces were not analyzed separately and the excretory products were not identified. However, it is apparent that part of the pentoses were either excreted directly or catabolized to excretory water-soluble compounds.

While the glycolysis of arabinose and xylose was retarded as evidenced by less radioactive CO_2 and more excreted radioactivity, the amounts of label retained in the carcass were greater than for glucose. The low values of radioactivity in the glycogen appear to be due to the dilution of the incorporated label by inherent and undepleted amount of liver glycogen from the unfasted birds at the time of injection of labeled sugars. However, it is evident that small amounts of label were incorporated into glycogen due to pentoses. These results lend support to earlier data (1) showing substantial metabolizable energy values of arabinose and xylose and confirm that these sugars are used metabolically.

Kohn et al. (12) observed in rats that about one-half of the intraperitoneally administered D-glucose-1-^{14}C was not accounted for in metabolic products. The authors suggested that the radioactivity retained in the carcass was converted primarily to macromolecular compounds. A recent report (13) has indicated that particles from the oviducts of hens have the ability to incorporate intact xylose molecules into tissue glycoprotein. Biosynthesis of UDP-xylose by decarboxylation of UDP-glucuronic acid has been demonstrated in avian species (14). Considerable radioactivity from each administered sugar was retained in the carcass and it is likely, in view of the observed slow oxidation to CO_2, that most of the retained radioactivity is incorporated as such into glycoprotein, protein, or acid mucopolysaccharides.

In bacteria isomerization of D-xylose to D-xylulose and enzymatic phosphorylation of D-xylulose to D-xylulose-5-phosphate by ATP has been reported (15, 16). The in-

[11] Wagh, P. V. 1965 Ph.D. Thesis, University of Minnesota, St. Paul.

corporation of a small amount of the label into expired CO_2, therefore, suggests that in chicks an isomerase may be present which by isomerization and phosphorylation would move D-xylose to enter in the pentose phosphate pathway.

Existence of an enzyme converting L-arabinose to L-arabitol has been suggested (17). The pathway of conversion of L-arabitol → L-xylulose → D-xylitol → D-xylulose (18–20) permitting L-arabinose to slowly oxidize to CO_2 is known. As an alternative, the conversion of L-arabinofuranose → L-arabano-γ-lactone → L-arabonic acid → α-ketoglutarate has been demonstrated in a bacterial system (21) providing a potential pathway for the catabolism of L-arabinose to CO_2.

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LITERATURE CITED

1. Wagh, P. V., and P. E. Waibel 1966 Metabolizability and nutritional implications of L-arabinose and D-xylose for chicks. J. Nutr., *90:* 207.
2. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York.
3. Wagh, P. V., and P. E. Waibel 1967 Alimentary absorption of L-arabinose and D-xylose in chicks. Proc. Soc. Exp. Biol. Med., *124:* 421.
4. Hamilton, P. B. 1962 Ion exchange chromatography of amino acids-micro-determination of free amino acids in serum. Ann. N. Y. Acad. Sci., *102:* 55.
5. Trevelyan, W. E., D. P. Procter and J. S. Harrison 1950 Detection of sugars on paper chromatograms. Nature, *166:* 444.
6. Huggett, A. S., and D. A. Nixon 1957 Use of glucose oxidase, peroxidase and o-dianisidine in determination of blood and urinary glucose. Lancet, *2:* 368.
7. Tolbert, B. M., M. Kirk and E. M. Baker 1956 Continuous $^{14}CO_2$ and CO_2 excretion studies in experimental animals. Amer. J. Physiol., *185:* 269.
8. Stetten, D., and G. E. Boxer 1944 Isolation of liver glycogen. J. Biol. Chem., *155:* 231.
9. Weimer, H. E. 1961 The effect of protein depletion and repletion on the concentration and distribution of serum proteins and protein-bound carbohydrates. Ann. N. Y. Acad. Sci., *94:* 225.
10. Eckel, R. E., C. E. Pope, II and J. E. C. Norris 1954 Lysine as a muscle cation in potassium deficiency. Arch. Biochem. Biophys., *52:* 293.
11. Wyngaarden, J. B., S. Segal and J. B. Foley 1957 Physiological disposition and metabolic fate of infused pentoses in man. J. Clin. Invest., *36:* 1395.
12. Kohn, P., R. J. Winzler and R. C. Hoffman 1962 Metabolism of D-glucosamine and N-acetyl-D-glucosamine in the intact rat. J. Biol. Chem., *237:* 304.
13. Grebner, E. E., C. W. Hall and E. F. Neufeld 1966 Incorporation of D-xylose-C^{14} into glycoprotein by particles from hen oviduct. Biochem. Biophys. Res. Commun., *22:* 672.
14. Bdolah, A., and D. S. Feingold 1965 Decarboxylation of uridine diphosphate-D-glucuronic acid by an enzyme preparation from hen oviduct. Biochem. Biophys. Res. Commun., *21:* 543.
15. Mitsuhashi, S., and J. O. Lampen 1953 Conversion of D-xylose to D-xylulose in extracts of *Lactobacillus pentosus.* J. Biol. Chem., *214:* 1011.
16. Stumpf, P. K., and B. L. Horecker 1956 The role of xylulose-5-phosphate in xylose metabolism of *Lactobacillus pentosus.* J. Biol. Chem., *218:* 753.
17. Touster, O., and S. O. Harewell 1958 The isolation of L-arabitol from pentosuric urine. J. Biol. Chem., *230:* 1031.
18. Ching, C., and S. G. Knight 1961 L-Arabinose metabolism by cell-free extracts of *Penicillium chrysogenum.* Biochim. Biophys. Acta, *46:* 271.
19. Hollman, S., and O. Touster 1957 The L-xylulose-xylitol enzyme and other polyol dehydrogenases of guinea pig liver mitochondria. J. Biol. Chem., *225:* 87.
20. McCormick, D. B., and O. Touster 1957 The conversion in vivo of xylitol to glycogen via the pentose phosphate pathway. J. Biol. Chem., *229:* 451.
21. Weimberg, R., and M. Duodoroff 1955 The oxidation of L-arabinose by *Pseudomonas saccharophila.* J. Biol. Chem., *217:* 607.

Albumin Content of Rat Hepatic Cells at Different Levels of Protein Intake [1]

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ABSTRACT A study was carried out to determine whether the protein content of the diet affects serum albumin synthesis by regulating the number of liver cells actively making albumin. A fluorescent anti-serum was prepared against rat albumin and used for identification of liver cells containing albumin. The number of albumin-containing cells in the liver was greatest when animals were fed a diet rich in protein; the number was at an intermediate level when maintained with a stock diet; and the number was very infrequent when the diet was devoid of protein. This observation is consistent with the effects of feeding different levels of protein on serum albumin turnover, on the protein content of the liver, and on the distribution of albumin in the liver cell.

Serum albumin is formed exclusively in in the liver (1, 2). Its identification within individual hepatocytes has been made possible by using the fluorescent antibody technique introduced by Coons and Kaplan (3). In this procedure, an antibody is prepared which is specific for a single protein; the antibody is then coupled to a dye that fluoresces in ultraviolet light. Thus, it becomes possible to identify the location of a protein in individual cells by treating sections of the tissue with the fluorescent antibody and thereafter viewing them by ultraviolet microscopy. Gitlin and co-workers (4) used this procedure to study the tissue distribution of several human plasma proteins. More recently, Hamashima et al. (5) improved the procedure for obtaining fluorescein-conjugated anti-albumin globulin and for fixing the albumin in the tissue samples and used this improved technique to localize albumin in human liver cells obtained at operation. The cytoplasm of some hepatocytes was found to stain brightly, while that of others did not. In consequence, they suggest that synthesis of albumin is an all-or-none process engaged in by only a proportion of the hepatocytes at a time. If this is so, the rate of albumin synthesis could be varied by increasing or decreasing the number of active cells.

It is now well-established that the metabolism of serum albumin is affected by dietary protein intake. For example, the feeding of a protein-deficient diet results in a reduction in the concentration of albumin in the plasma (6, 7). This occurs despite a retardation in the rate of albumin breakdown during protein depletion (8, 9). Consequently, there must be an even greater reduction in the rate of albumin synthesis in the livers of animals receiving an inadequate protein intake. It would be expected therefore that animals fed a diet deficient in protein would show few albumin-containing hepatocytes, whereas animals given a high intake of protein would have many hepatocytes exhibiting fluorescence when treated with the fluorescent antibody to plasma albumin. In the experiments described in the present report, this has been confirmed in rats fed different levels of protein.

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EXPERIMENTAL

Animals and diets. Young adult male albino rats weighing 140 to 200 g were selected from the department colony (Wistar strain). Control animals were fed ad libitum a stock diet containing 16% protein. Other groups were fed either a protein-rich diet providing 28% casein or a protein-free diet at the same calorie intake, namely 1450 kcal/m^2/day (8). The adequate diet contained 28.5% casein, 41.8% potato starch, and 15% glucose; the protein-free diet provided 64% starch and 21% glucose, but no protein. Both diets also included 5% margarine fat and 10% of a vitamin-mineral-roughage mixture.[5] The diets were fed for 4 days and then the animals were anesthetized with ether, blood was removed from the inferior vena cava into heparinized tubes and the liver excised.

Preparation of tissue sections. The procedure of Hamashima et al. (5) was used. Small samples of liver were fixed in 95% ethanol containing 1% acetic acid at 4° overnight, then dehydrated, cleared, and embedded in paraffin as described by Sainte-Marie (10). Sections 4 μ thick were cut, dried, deparaffined, rehydrated and stained with fluorescent antibody for one-half to one hour. After washing with phosphate-buffered saline, the sections were mounted in glycerol, or after dehydration in "UV inert" medium,[6] before being examined in the fluorescence microscope. Other sections were stained with hematoxylin and eosin.

Preparation of fluorescent antibody to rat albumin. Albino rabbits were immunized against whole rat plasma by the procedure of Benjamin and Weimer (11). Rat albumin was prepared by chromatography on DEAE-Sephadex of a crude fraction obtained from rat plasma by half-saturation with ammonium sulphate (12). The final preparation of albumin had a molecular weight of 66,000 and gave a single band after immunoelectrophoresis on agar gel (13) and by the Ouchterlony agar diffusion plate technique (13) when tested against antibody to whole rat plasma. This pure albumin was then used in the preparation of a specific anti-rat albumin in rabbits by the same procedure described earlier. After completion of the immunization program, a crude globulin fraction was obtained from the anti-rat albumin antiserum by half-saturation with ammonium sulphate. This crude globulin was then conjugated with fluorescein isothiocyanate using the procedure of Marshall et al. (14). Unconjugated fluorescent material was removed by dialysis against phosphate-buffered saline at pH 7, followed by passage through a G50 Sephadex column. To reduce nonspecific staining, the fluorescent antibody was purified by column chromatography using DEAE-cellulose (15). The purity of the fluorescent globulin was confirmed by precipitin tests, diffusion in agar and the fluorescence

[5] This mixture contained per 100 g: (in grams) starch, 41.0; cellulose (Wood Flock, Brown Company, Berlin, N. H.), 11.3; cod liver oil, 11.3; wheat germ oil, 2.7; sodium chloride, 8.5; dicalcium phosphate, 8.4; potassium citrate, 6.6; calcium carbonate, 3.9; dipotassium phosphate, 1.8; choline chloride, 3.6; and (in milligrams) magnesium carbonate, 950; ferric citrate, 380; manganous sulfate, 30; copper sulfate, 4; potassium aluminum sulfate, 2; cobalt chloride, 2; potassium iodide, 1; zinc carbonate, 1; sodium fluoride, 0.02; inositol, 90; p-aminobenzoic acid, 45; calcium pantothenate, 18; nicotinic acid, 9; thiamine·HCl, 2; riboflavin, 2; and pyridoxine·HCl, 2.

[6] G. T. Gurr, Ltd., London.

TABLE 1

Concentrations of plasma protein, plasma albumin and liver protein in rats fed stock diet (16% protein), a 28% protein diet and a protein-free diet

Diet	Plasma protein concn		Liver protein concn [3]
	Total [1]	Albumin [2]	
	g/100 ml		*mg/g*
Stock	6.5 ± 0.51 [4]	3.34 ± 0.81	184 ± 1.0
28% protein	6.6 ± 0.61	3.53 ± 0.64	209 ± 14.7
0% protein	6.3 ± 0.41	2.90 ± 0.52	166 ± 8.0

[1] Mean of 9 rats/dietary group. There are no significant differences between groups ($P > 0.05$).
[2] Mean of 9 rats/dietary group. Plasma albumin levels with the protein-free diet are significantly lower than with the other 2 diets ($P < 0.02$).
[3] Mean of 3 rats/dietary group. The concentration of liver protein is significantly lower with the protein-free diet than on the 28% protein diet ($P < 0.05$).
[4] SD.

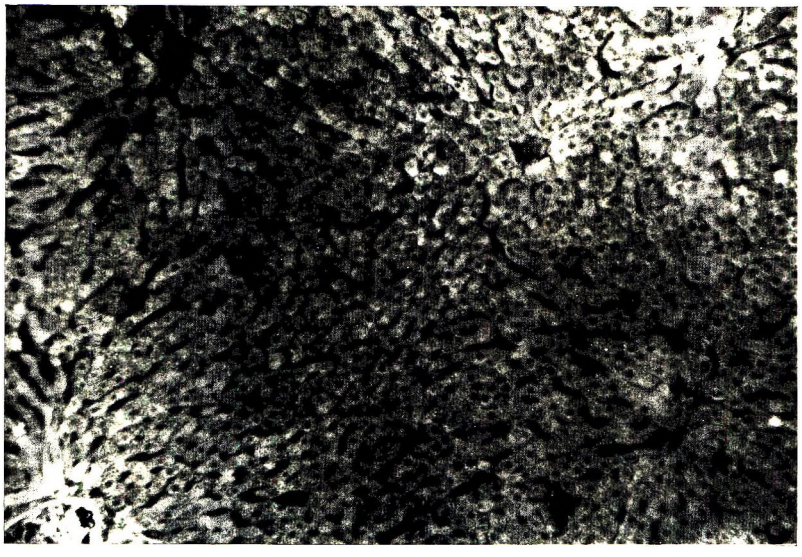

Figure 1a

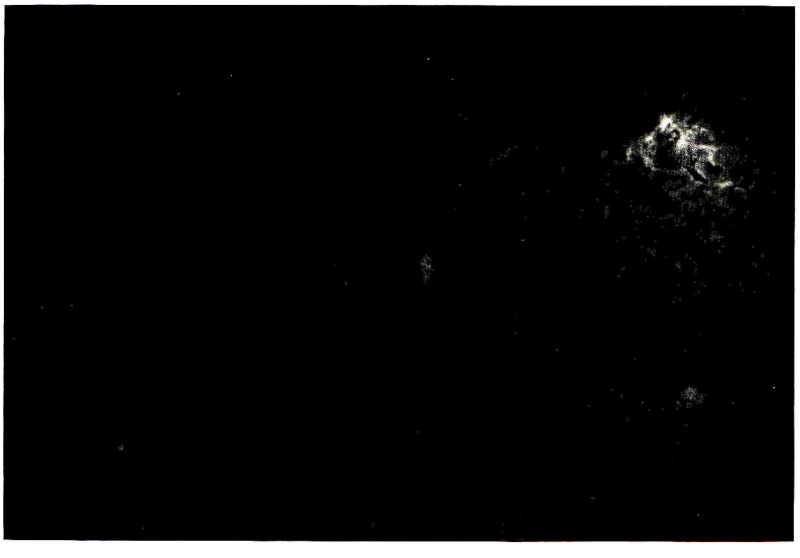

Figure 1b

Fig. 1 Photomicrographs of sections obtained from the livers of (a) rats fed a 28% protein diet and (b) rats fed a 0% protein diet and treated with fluorescent anti-albumin. Low power (× 44). An Ortholux microscope with Orthomat camera and photoelectric exposure meter (E. Leitz, Wetzlar) was used with Kodak Linograph film (35 mm). All exposures were made automatically at the same instrument setting and the prints were produced under identical standard conditions.

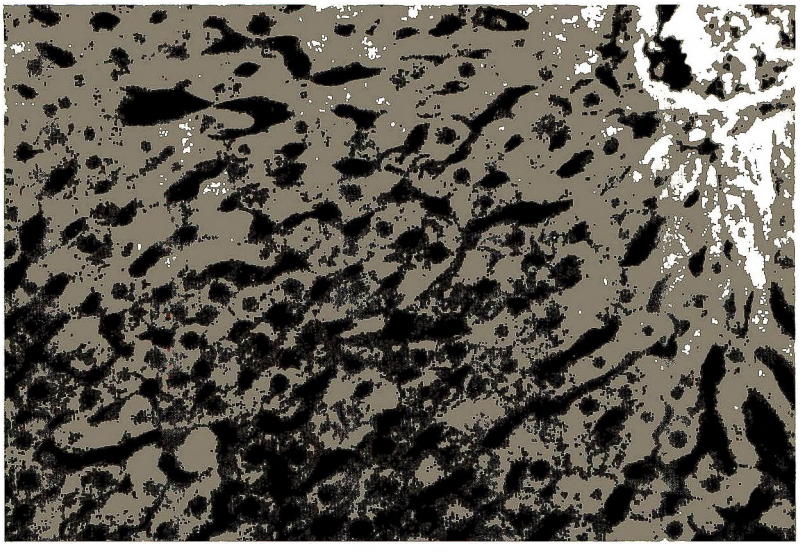

Figure 2a

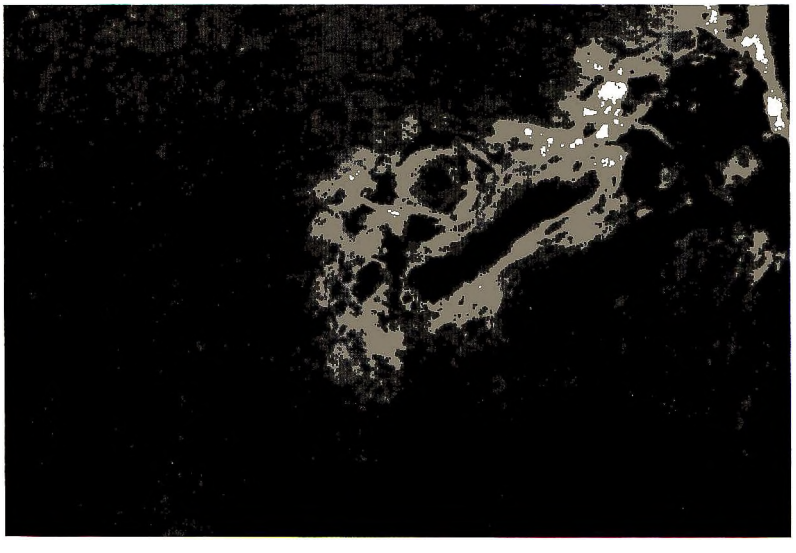

Figure 2b

Fig. 2 Same as figure 1, but under higher power magnification (× 109).

spectrum obtained in the Aminco-Bowman Spectrofluorimeter (16).

Analytical methods. Total liver protein was estimated on a sample of an aqueous homogenate of liver by the method of Lowry et al. (17). Total plasma protein and plasma albumin were determined by the procedure of Gornall et al. (18). The proportion of plasma albumin was verified by the paper electrophoretic method of Kenny (19).

RESULTS

Deprivation of protein or feeding a protein-rich diet for 4 days had no significant effect on the total concentration of pro-

tein in the plasma (table 1). However, this short period did lead to a significant reduction in plasma albumin concentration in the group deprived of protein (table 1). For comparison, the change in the liver total protein content in animals from one experimental series is also shown in table 1. The groups fed the 28% and the 0% protein diets showed a significant 17% difference in liver protein level (t test, $0.05>P>0.02$). In contrast with these small but significant changes, the number of albumin-containing cells in the liver was markedly affected by diet. The appearance of the fluorescent-stained fields (fig. 1, 2) was similar to the fields obtained by Hamashima et al. (5) in sections from human subjects. Sections cut from the livers of animals fed the 28% protein diet were highly fluorescent and the nuclei of almost all the hepatocytes were clearly visible as dark areas against the fluorescent cytoplasm. The degree of fluorescence varied considerably but was most marked along the sinusoids. In contrast, there was very little fluorescent staining in sections cut from the livers of protein-deficient animals. There was only an occasional isolated fluorescent cell, again with an increase in albumin-containing cells close to the sinusoids. The appearance of sections prepared from control animals fed the stock diet was intermediate between these extremes.

An attempt was made to place these observations on a quantitative basis, by enumerating the numbers of fluorescent cells per microscopic field. In an initial experiment, 2 groups were compared, one fed 28% protein and the other the stock diet and the number of fluorescent cells per high power field was counted. The average number of fluorescent cells per field in the 28% protein groups was 143 (SD± 13.4); for the controls fed stock diet the mean number of fluorescent cells per field was 24 (SD±8.5), a difference that is statistically highly significant. To make a more thorough evaluation of the influence of protein intake, the percentage of fluorescent cells was then enumerated in several fields for rats receiving each of the 3 diets fed. Table 2 evaluates the effect of diet on the proportion of liver cells with visible albumin. It is apparent that the percentages of hepatic cells that fluoresce are related to the protein content of the diet.

To eliminate the possibility that these findings resulted from nonspecific binding of fluorescent material, some sections from animals fed either 28% or 0% protein were treated with fluorescent-labeled anti-albumin which had been mixed with rat albumin before staining to neutralize the antibody. Sections prepared in this way showed no fluorescent material.

DISCUSSION

The picture presented by the present studies (table 1) confirm earlier demonstrations (6, 7) that the level of plasma albumin is a more sensitive index than the level of total plasma protein to deficiency of dietary protein. Protein deficiency reduced both the plasma albumin level and the total protein concentration in the liver to approximately 20% below the level obtained with animals fed the high protein diet. The levels observed with animals fed the stock diet (16% protein) were intermediate between these extremes. This picture can be compared with the more marked changes in histochemically identifiable albumin in the hepatocytes of animals fed these different diets (table 2; figs. 1, 2). At the highest level of protein intake, the majority of cells stained for albumin, whereas with the protein-

TABLE 2

Dietary protein content and frequency of fluorescent cells [1]

Diet	Total no. of cells examined	Parenchymal cells	Kupffer cells	Fluorescent cells
		%	%	%
Stock	284	75	25	10
28% protein	296	77	23	60
0% protein	341	74	26	— [2]

[1] Ten fields counted/diet.
[2] Too infrequent to measure accurately.

free diet such cells were infrequent. This could imply that albumin biosynthesis is grossly reduced in the liver of the protein-deficient animal by restricting the number of active cells, as suggested by Hamashima et al. (5). However, it is also possible that hepatocytes containing identifiable albumin do so because of accumulation of albumin within the vesicles of the endoplasmic reticulum as a result of vigorous secretion of this protein into this intracellular transport system (20). Fluorescent albumin within the cell may therefore represent mainly the albumin within these vesicles and not necessarily albumin only at the site of synthesis. It would not be unexpected if the total amount of albumin undergoing intracellular transport fell sharply during depletion. Hoffenberg et al. (21) observed in human volunteers subjected to protein deficiency that the extravascular albumin pool was more rapidly affected than the intravascular pool; albumin within the vesicles of the endoplasmic reticulum is one form of extravascular albumin. These considerations show that the interpretation of immunofluorescence studies of hepatocyte albumin content is not a simple one.

LITERATURE CITED

1. Miller, L. L., and W. F. Bale 1954 Synthesis of all plasma protein fractions except gamma globulins by the liver. The use of zone electrophoresis and lysine-C_{14} to define the plasma proteins synthesised by the isolated perfused liver. J. Exp. Med., *99:* 125.
2. McFarlane, A. S. 1957 Use of labelled plasma proteins in the study of nutritional problems. Progr. Biophys. Chem., *7:* 115.
3. Coons, A. H., and M. H. Kaplan 1950 Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med., *91:* 1.
4. Gitlin, D., B. H. Landing and A. Whipple 1953 The localization of homologous plasma proteins in the tissues of young human beings as demonstrated with fluorescent antibodies. J. Exp. Med., *97:* 163.
5. Hamashima, Y., J. G. Harter and A. H. Coons 1964 The localization of albumin and fibrinogen in human liver cells. J. Cell. Comp. Physiol, *20:* 271.
6. Platt, B. S., C. R. C. Heard and R. J. C. Stewart 1964 Experimental protein-calorie deficiency. In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison. Academic Press, New York, p. 446.
7. Viteri, F., M. Béhar, G. Arroyave and N. S. Scrimshaw 1964 Clinical aspects of protein malnutrition. In: Mammalian Protein Metabolism, vol. 2, eds., H. N. Munro and J. B. Allison. Academic Press, New York, p. 523.
8. Fleck, A., and H. N. Munro 1963 Protein metabolism after injury. Metab. Clin. Exp., *12:* 783.
9. MacFarlane, A. S. 1964 Metabolism of plasma proteins. In: Mammalian Protein Metabolism, vol. 1, eds., H. N. Munro and J. B. Allison. Academic Press, New York, p. 297.
10. Sainte-Marie, G. 1962 A paraffin embedding technique for studies employing immunofluorescence. J. Histochem. Cytochem., *10:* 250.
11. Benjamin, D. C., and H. E. Weimer 1963 The antigenic composition of rat serum: an electrophoretic study. J. Immunol., *91:* 331.
12. Fleck, A., N. Chandra, G. Moffet, J. Crossan and T. A. Douglas 1966 The separation of serum proteins by column chromatography. Proc. Assoc. Clin. Biochem., *4:* 117.
13. Crowle, A. J. 1961 Immunodiffusion. Academic Press, New York.
14. Marshall, J. D., W. C. Evelund and C. W. Smith 1958 Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technic with a modification of its application. Proc. Soc. Exp. Biol. Med., *98:* 898.
15. McDevitt, H. O., J. H. Peters, L. W. Pollard, J. G. Harter and A. H. Coons 1963 Purification and analysis of fluorescein-labelled antisera by column chromatography. J. Immunol., *90:* 634.
16. Nairn, R. C. 1964 Fluorescent Protein Tracing, ed. 2. E. and S. Livingston Ltd. Edinburgh.
17. Lowry, O. H., N. J. Rosenbrough, A. L. Farr and R. J. Randall 1951 Protein estimation with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
18. Gornall, A. G., C. J. Bardawill and M. M. David 1949 Determination of serum proteins by means of the biuret reaction. J. Biol. Chem., *177:* 751.
19. Kenny, A. P. 1965 Preparation of a cam for the scanning of paper electrophorograms on the Chromascan densitometer. Clin. Chim. Acta, *11:* 170.
20. Peters, T., Jr. 1962 The biosynthesis of rat serum albumin. J. Biol. Chem., *237:* 1186.
21. Hoffenberg, R., E. Black and J. F. Broch 1966 Albumin and gamma-globulin tracer studies in protein depletion states. J. Clin. Invest., *45:* 143.

Effect of Threonine on Tyrosine Metabolism of Rats Fed a Low Protein Diet

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ABSTRACT The alleviation of tyrosine toxicity obtained by the addition of threonine to a 3% tyrosine low protein diet was studied to determine the mode of action of threonine. Rats fed a 3% L-tyrosine and 1.25% L-threonine low protein diet had a lower plasma tyrosine concentration as compared with rats fed a 3% L-tyrosine low protein diet. Injection of these rats with L-tyrosine-U-^{14}C indicated that the effect of threonine was not to increase the catabolism of tyrosine to $^{14}CO_2$ but to increase the excretion of radioactivity in the urine and the incorporation of radioactive tyrosine into plasma and liver proteins. In rats receiving an excess of tyrosine or threonine or both amino acids, the incorporation of DL-alanine-1-^{14}C into tissue proteins was also increased over the control rats indicating a general stimulation of amino acid incorporation into proteins.

Recently Harper and co-workers (1) have shown that the addition of 1.25% of L-threonine to a high tyrosine, low protein diet prevented the appearance of the typical symtoms of tyrosine toxicity and improved growth. The addition of threonine to the diet reduced the concentration of plasma tyrosine (2) and a stimulation by threonine of the oxidation of tyrosine in the liver was suggested as a possible explanation for the reduced tyrosine concentration in plasma. The present report describes a study of the metabolism of radioactive tyrosine in rats fed high threonine and high tyrosine diets.

EXPERIMENTAL

Young 50-g male albino rats of the Wistar strain were used. Food and water were supplied ad libitum. The basal diet had the following composition: (in % dry weight) casein, 8.0; starch, 75.0: hydrogenated vegetable oil, 13.0; salt mixture and vitamin supplement, 4. The salts and vitamins have been described previously (3) and were used in the same amounts. The niacin content of the diets was 0.06 g/100 g. The diets used were prepared by adding to the basal diet 0.2% DL-methionine (diet 1); 0.2 DL-methionine and 3% L-tyrosine (diet 2); 0.2% DL-methionine and 1.25% L-threonine (diet 3); or 0.2% DL-methionine, 3% L-tyrosine and 1.25% L-threonine (diet 4). Four groups of 8 rats each were fed ad libitum the diets described. After 21 days the rats were injected intraperitoneally 0.5 ml of isotonic sodium chloride containing 1 mg of L-tyrosine-U-^{14}C or DL-alanine-1-^{14}C (specific activity 1 μCi/mg).[1] The excretion of radioactivity in respiratory CO_2 and in urine and the incorporation of ^{14}C-amino acids in tissue proteins were determined as described previously (3).

RESULTS

When rats maintained for 21 days with the 4 experimental diets, the 3% L-tyrosine diet (diet 2) and the 3% L-tyrosine and 1.25% L-threonine diet (diet 4) depressed growth (table 1) but not as markedly as reported earlier (1). Excess threonine in the diets (diets 3 and 4) decreased the plasma tyrosine level as compared with the level in rats fed diets 1 and 20. Here again the decrease in tyrosine level was not as great as that reported elsewhere (2). Eye and jaw lesions were observed in rats fed diet 2 after 7 days but very few lesions were observed in rats fed diet 4, even after 21 days.

Tracer doses of L-tyrosine-^{14}C were injected into rats fed the 4 experimental diets. Excretion of radioactivity in respiratory CO_2 and in urine was determined during the 6 hours following injection (table 2). In rats fed a high tyrosine diet

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[1] Radioactive amino acids were obtained from the Atomic Energy of Canada, Commercial Products Division, Ottawa, Canada.

TABLE 1

Effect of 3% L-tyrosine and 1.25% L-threonine on plasma amino acids and growth [1]

	Dietary treatment	Body wt gain after 21 days	Plasma amino acid concn [2]		
			Tyrosine	Threonine	Alanine
		g	*μmoles/100 ml plasma*		
1	Control	55.9 ± 1.8 [3]	12.0	10.5	79.7
2	3% L-tyrosine	40.4 ± 2.6	47.0	9.5	64.2
3	1.25% L-threonine	52.0 ± 1.4	7.8	16.4	80.4
4	3% L-tyrosine + 1.25% L-threonine	46.4 ± 2.2	27.0	6.6	67.0

[1] Pooled samples for all 4 groups of 4 rats.
[2] Amino acids were analyzed on an automatic amino acid analyzer after precipitation of the plasma proteins with 3% salicylsuphonic acid.
[3] Mean ± SE.

TABLE 2

Excretion of radioactivity in CO_2 and urine of rats injected with radioactive tyrosine and alanine [1]

Substance injected [2]	Recovered in	Time elapsed	Excretion of radioactivity, cumulative percentages			
			Diet 1	Diet 2	Diet 3	Diet 4
		hours				
L-Tyrosine-U-^{14}C	CO_2	1	10.1 ± 1.2 [3]	12.3 ± 1.6	8.2 ± 1.9	10.6 ± 1.7
	CO_2	6	24.6 ± 1.9	39.6 ± 2.1	18.5 ± 2.4	36.0 ± 3.2
	urine	6	2.9 ± 0.8	4.5 ± 1.2	5.4 ± 1.1	5.8 ± 1.4
DL-Alanine-1-^{14}C	CO_2	1	39.6 ± 3.4	40.6 ± 1.8	38.2 ± 3.8	41.0 ± 1.6
	CO_2	6	74.9 ± 2.7	73.0 ± 3.0	74.7 ± 4.1	75.6 ± 2.9
	urine	6	1.4 ± 0.2	1.2 ± 0.2	0.8 ± 0.1	1.0 ± 0.1

[1] Six rats were used in each experiment. Diets are described in the Experimental section. Rats were fed ad libitum for 21 days before administration of isotopes.
[2] Isotonic NaCl (0.5 ml) containing 1 mg of the radioactive substance (1 μCi/mg) was injected intraperitoneally.
[3] Mean ± SE of mean of cumulative percentages of administered dose excreted.

(diets 2 or 4) the catabolism of radioactive tyrosine to $^{14}CO_2$ and the excretion of radioactivity in the urine was increased. Stimulation of the catabolism of radioactive phenylalanine and radioactive tyrosine in rats fed a high phenylalanine diet has been reported (3).

When an excess of threonine was present as well as an excess of tyrosine (diet 4) the increase in excretion of radioactivity was not as great but the differences between the 2 treatments (diets 2 and 4) were not significant. In rats fed a high threonine diet (diet 3) the excretion of radioactivity in CO_2 was slightly lower than in the control group (diet 1). The difference was significant only after 6 hours. The excretion of radioactivity in urine, however, was higher in rats fed diet 3 than in rats fed diet 1. These results indicate that the lowering of the tyrosine in plasma of rats observed both in the present study and previously (2) was not the result of stimulation by threonine of tyrosine oxidation. In addition, table 2 shows that under the same experimental conditions the catabolism of radioactive alanine to CO_2 and the excretion of radioactivity in urine was not modified significantly by an excess of tyrosine or threonine in the diet.

The incorporation of ^{14}C coming from injected tyrosine or alanine into trichloroacetic acid-insoluble plasma and liver proteins is reported in table 3. In rats fed the high tyrosine diet (diet 2), the incorporation of ^{14}C-tyrosine into proteins was slightly lower than in control rats. This was not unexpected as it has been reported that in rats fed a high phenylalanine diet, the incorporation of radioactive phenylalanine into tissue proteins is lower than in control rats (3). This is probably the result of the dilution of the injected radioactive amino acid by the excess of the same amino acid in the plasma. In rats fed a high threonine diet (diet 3), the incorporation of ^{14}C-tyrosine in tissue proteins was much higher than in control rats. In rats fed the high tyrosine and threonine

TABLE 3

Distribution of radioactivity in solubilized tissue proteins of rats injected with radioactive tyrosine and alanine [1]

Substance injected [2]	Tissue	Diet 1	Diet 2	Diet 3	Diet 4
		count/min/mg protein			
L-Tyrosine-U-^{14}C	liver	5.1 ± 0.7 [3]	3.6 ± 0.3	11.4 ± 0.8	4.8 ± 0.7
	plasma	14.4 ± 1.2	9.0 ± 0.4	26.7 ± 1.2	14.7 ± 0.9
DL-Alanine-1-^{14}C	liver	5.2 ± 0.2	8.5 ± 0.2	9.1 ± 0.1	7.0 ± 0.8
	plasma	10.6 ± 0.6	16.1 ± 0.4	19.6 ± 1.0	12.7 ± 0.3

[1] Four rats were used in each experiment. Diets were fed ad libitum for 21 days before administration of isotope. Proteins were obtained and counted as described previously (3).
[2] Isotonic NaCl (0.5 ml) containing 1 mg of the radioactive substance (1 μCi/mg) was injected intraperitoneally, and represented 10^6 count/min.
[3] Mean ± SE.

diet 4, the effect of threonine was to increase the incorporation of radioactive tyrosine into proteins to the same level as in the control rats and therefore to prevent the decrease in incorporation observed in rats fed diet 2. Under the same experimental conditions, an excess of tyrosine or threonine in the diet stimulated the incorporation of radioactive alanine in plasma and liver proteins. A decrease in alanine plasma concentration was also observed in some cases (table 1).

DISCUSSION

The results presented here indicate that the decrease in plasma tyrosine concentration in rats fed a 1.25% L-threonine diet (diets 1 and 2) was not the result of stimulation of the oxidation of tyrosine. On the contrary, the catabolism of tyrosine to CO_2 was slightly decreased in rats having a high plasma threonine concentration. That there is no stimulation of amino acid oxidative catabolism in general under these conditions is indicated by the unchanged excretion of radioactivity in CO_2 after injection of radioactive alanine.

The decrease in plasma tyrosine concentration in the presence of excess threonine was due 1) to a slight increase in the excretion of radioactivity from ^{14}C-tyrosine in the urine, and 2) to an increase in the incorporation of radioactive tyrosine in plasma and liver proteins. This increase in incorporation of tyrosine into proteins was the result of an increase in protein synthesis as indicated by an increase in the incorporation of ^{14}C-alanine in proteins under the same conditions. This more efficient use of amino acids for protein formation when rats are fed those unbalanced diets is comparable to the increased incorporation into proteins of the most limiting amino acid observed by Yoshida and co-workers (4) in cases of amino-acid imbalance.

In diet 1, threonine is the limiting amino acid. Addition of threonine to the diet provided a better-balanced diet and the rats fed diet 3 incorporated tyrosine and alanine into proteins to a greater extent than the control rats (diet 1). Addition of tyrosine to diet 1 also provided a better-balanced diet as shown by an increased incorporation of radioactive alanine in rats fed diet 2. However, the beneficial effect of the addition of tyrosine or threonine to diet 1 on the utilization of amino acids for protein synthesis was not additive. When both tyrosine and threonine were added to diet 1, the increase in incorporation of alanine in the proteins of rats fed diet 4 was not as great as the one obtained in rats fed diet 2 or diet 3. The addition of an excess of these 2 amino acids together may very well produce a large increase in the requirement for some other essential amino acids present in a low concentration in the diet, thus limiting the utilization of amino acids for protein synthesis.

The increased excretion of radioactivity in the urine of rats injected with L-tyrosine-^{14}C could be due to the presence in the plasma of a large excess of a given amino acid such as tyrosine or threonine.[2] Increased excretion of radioactivity in the urine of rats injected with radioactive

[2] Cullen, A. M., and H. N. Christensen 1966 Effects on amino acid distribution of the injection of model amino acids. Federation Proc., 25: 541 (abstract).

phenylalanine or tyrosine and maintained with a high phenylalanine diet has been observed previously (3). However, no increase in the excretion of radioactivity was observed when the rats were injected with radioactive alanine. This could indicate some competition between threonine and tyrosine during reabsorption in the renal tubules, competition which would not involve alanine.

ACKNOWLEDGMENT

The author thanks the undergraduate students in biology, 1966–1967, for helpful technical assistance.

LITERATURE CITED

1. Alam, S. Q., R. V. Becker, W. P. Stucki, Q. R. Rogers and A. E. Harper 1966 Effect of threonine on the toxicity of excess tyrosine and cataract formation in the rat. J. Nutr., *89:* 91.
2. Alam, S. Q., Q. R. Rogers and A. E. Harper 1966 Effect of tyrosine and threonine on free amino acids in plasma, liver, muscle and eye in the rat. J. Nutr., *89:* 97.
3. Godin, C., and G. Dolan 1966 Metabolism of radioactive phenylalanine in rats with different dietary intakes of phenylalanine. J. Nutr., *90:* 284.
4. Yoshida, A., P. M. B. Leung, Q. R. Rogers and A. E. Harper 1966 Effect of amino acid imbalance on the fate of the limiting amino acid. J. Nutr., *89:* 80.

Proceedings of the Thirty-first Annual Meeting of the American Institute of Nutrition

SHERATON-BLACKSTONE HOTEL, CHICAGO, ILLINOIS
APRIL 16–21, 1967

COUNCIL MEETINGS

The Council of the American Institute of Nutrition met Saturday evening, April 15, and Sunday morning and evening, April 16. The actions of the Council were presented at the Institute business meetings and are included in the report of those meetings, published below.

SCIENTIFIC SESSIONS

A total of 351 abstracts of papers was accepted by the AIN; 55 were transferred to other societies or intersociety sessions, 59 were accepted from other societies, making a total of 355 papers programed by the Institute. These were arranged into 27 regular AIN and 5 intersociety (Atherosclerosis) sessions. In addition, two informal conferences were held, Poultry Nutrition and Ruminant Nutrition. Six AIN symposia were programed this year, scheduled in pairs with no concurrent short-paper sessions.

1. Nutrition and Bone Loss
2. Malabsorption
3. Geographic Nutrition
4. Nutrition and Work Performance
5. Nutrition and Prenatal Development
6. Alcohol, Metabolism and Liver Disease

BUSINESS MEETINGS

Business meetings were held on Tuesday, April 18 and Thursday, April 20. Dr. A. E. Schaefer presided at both meetings.

I. *Proceedings of 1966 Meeting*

The Proceedings as published in the Journal of Nutrition, *90:* 101–114, 1966, were approved.

II. *Elections*

The 658 ballots were counted by Drs. M. S. Read and C. J. Ackerman. The following were elected:

President-Elect:
Richard H. Barnes
Councilor:
Howerde E. Sauberlich
Nominating Committee:
J. K. Loosli, Chairman
Doris H. Calloway
Philip L. Harris
Fred A. Kummerow
Calvin W. Woodruff

III. *Revision of AIN Constitution and Bylaws*

The revised AIN Constitution and Bylaws were approved by 592 in favor and 10 against. The newly adopted Constitution and Bylaws will appear in the 1967 Federation *Directory of Members.*

IV. *Membership*

As of April 15, 1967, there were 1097 members of the Institute: 966 active, 110 retired, and 21 honorary members, this being a net increase of 63 members since last year. Fourteen members retired during the year. The Clinical Division reports a total membership of 189.

Notice of the deaths of the following members was received since our last annual meeting:

Adelia M. Beeuwkes, June 23, 1966
H. Louise Campbell (Charter Member), February 27, 1967
W. H. Chambers (Charter Member), April 16, 1966
V. H. Cheldelin, August 23, 1966
E. B. Forbes (Charter Member), September 8, 1966
George J. Hamwi, February 14, 1967
B. W. Heywang, May 19, 1966
Rebecca B. Hubbell, November 3, 1966
I. S. Kleiner (Charter Member), June 15, 1966
D. A. Libby, January 31, 1967

E. J. Quinn (Charter Member), February 12, 1967
W. C. Supplee, July 25, 1966
D. W. Woolley, July 23, 1966

In accordance with the wishes expressed by the membership to shorten the business meetings, it was decided that resolutions on deceased Charter Members would not be read during the meetings but would be published as part of the Annual Proceedings for permanent record.

RESOLVED: That the American Institute of Nutrition assembled in Chicago, Illinois, at its annual meeting, April 18, 1967, place in its Proceedings for permanent record this statement of deep regret and sorrow at the loss by death of its Charter Member, *Dr. H. Louise Campbell,* on February 27, 1967, and further that special recognition be given to her contributions to the development of the science of nutrition.

Dr. Campbell was well-known for her research in connection with the longevity studies carried out in Dr. Henry C. Sherman's laboratory covering the progeny of over 50 generations of experimental animals. These animals were maintained throughout life on a simplified diet comparing the effects of two levels of milk on the length of life, fertility, vigor and general well-being. The immense accumulation of data over such a long period of time is without parallel. She was coauthor of many papers. Her high standards of work and her painstaking attention to details set an example for the many students and research associates who had the privilege of working with her.

RESOLVED: The American Institute of Nutrition assembled at Chicago at its annual meeting April 18, 1967, wishing to express its deep sense of loss and sorrow at the passing of one of its most distinguished founding members, *William Harold Chambers,* places this statement in its Proceedings for permanent record.

Dr. Chambers served as Secretary of the Federation of American Societies for Experimental Biology, and Managing Editor of *Federation Proceedings* in 1947 and 1948. He served as Assistant Editor to the *Journal of Nutrition* and on the editorial boards of *Physiological Reviews* and the *Journal of Applied Physiology.*

His greatest periods of scientific productivity were at Cornell University Medical College (1928–1947) and later at the Army Chemical Corps Medical Laboratories (1947–1960). His contributions to the understanding of the metabolic changes during fasting were classical studies.

Dr. Chambers' contributions to this world cannot be measured only by his scientific achievements. He was a patriot in the true sense of the word and served his country with acknowledged commendations in World Wars I and II.

His warmth and understanding made it a privilege to have worked with him. His example will remain with us for the future.

RESOLVED: That the American Institute of Nutrition, assembled at Chicago, Illinois, at its annual meeting, April 18, 1967, place the following statement in its Proceedings for permanent record to express its appreciation for the contributions of one of its distinguished Charter Members, *Ernest Browning Forbes,* Professor Emeritus of Animal Nutrition, Pennsylvania State University, who passed away at his home on September 8, 1966.

Professor Forbes received bachelor's degrees in Zoology and in Agriculture from the University of Illinois in 1897 and 1902 and his doctorate from the University of Missouri in 1908. While on the staff of the University of Missouri as assistant professor 1903–1907, he taught animal husbandry and initiated a series of studies in mineral metabolism which was to continue as his major research interest as Chief, Department of Nutrition at the Ohio Agricultural Experiment Station during the years 1907–1920. He served with the rank of Major as a Nutrition Officer in the AEF in France during World War I. In 1921 he was appointed Director of the Institute of Animal Nutrition, Pennsylvania State College and remained in this position until retirement as Professor Emeritus in 1946.

Among the honors which he received were selection as Honored Guest of the American Society of Animal Production in 1934 and election to foreign membership in the Royal Swedish Academy of Agriculture in 1940. He was a Charter Member of the American Society of Animal Production and a member and Chairman of the Committee on Animal Nutrition of the National Research Council.

Professor Forbes' early investigations on the mineral metabolism of swine and of dairy cattle were characterized by meticulous experimental procedures and the demonstration of the unavoidable mineral balances of early lactation. The same concern for careful and improved research procedures characterized the subsequent investigations that he and his associates at the Pennsylvania State College carried out on energy metabolism, the factors which influence energy utilization, and methods of evaluating the energy contribution of feeds.

Professor Forbes has left with us a legacy of thoughtfulness and consideration in his dealings with his fellow men and with a concept of the importance of persistence and careful experimentation in research.

RESOLVED: That the American Institute of Nutrition, assembled in Chicago, Illinois, at its annual meeting April 18, 1967, recognize the loss by death of one of its distinguished Charter Members, *Israel Simon Kleiner,* on June 10, 1966 and place this statement in its Proceedings for permanent record.

Dr. Kleiner was trained at Yale University, receiving the Ph.B. degree in 1906 and Ph.D. degree, under Professors Lafayette B. Mendel and F. P. Underhill, in 1909. After study in England, Sweden, and Germany, he was appointed Instructor in Biochemistry at Tulane University School of Medicine and then an

Assistant and an Associate Professor in Biochemistry at the Rockefeller Institute from 1910 to 1919. He was appointed Professor and Chairman of the Department of Biochemistry at New York Medical College in 1920, a position he held until he retired in 1959. He also served as Dean of the New York Medical College from 1921 to 1925 and as a Counsulting Chemist at Flower — Fifth Avenue Hospital. He was appointed Emeritus Professor of Biochemistry in 1963 and continued active work at the Medical College almost to the time of his death.

The areas of carbohydrate metabolism and its regulatory hormones, the vitamins (especially, ascorbic acid) and the enzymes of the gastrointestinal tract (particularly rennin and pepsin), were Dr. Kleiner's primary research interests. In 1915, he, with S. J. Meltzer, was among the first to demonstrate that some substance elaborated by the pancreas lowered the blood sugar when administered intravenously to diabetic dogs. This substance, present in simple aqueous emulsions of pancreatic tissue, was later shown to be insulin by the classic experiments of Banting and Best in 1922.

Dr. Kleiner was perhaps best known, however, as the author, and later coauthor with J. M. Orten, of the widely used textbook, *Human Biochemistry*. He had just completed his portion of the new seventh edition of this book at the time of his death.

RESOLVED: That the American Institute of Nutrition place in its Proceedings for permanent record the statement of sincere regret and sorrow at the loss by death of one of its distinguished Charter Members, *Edward J. Quinn,* on February 12, 1967.

Dr. Quinn was awarded a Ph.D. degree in chemistry from Columbia University where he also served as a member of the faculty for several years. He was associated with Professor H. C. Sherman during the early days of vitamin research. Many of his important contributions were concerned with the nutritional properties and stability of vitamins A and D and of the vitamin B complex. He also contributed to our knowledge of calcium and phosphorus metabolism.

Dr. Quinn served as Director of Research of the Maltine Company from 1930–1937 and as a vitamin specialist to Merck and Company, Inc., from 1937–1951. It was during this period that vitamins were made available on a commercial scale and Dr. Quinn contributed heavily to this important work. His many scientific contributions were made all the more impressive by his warm personal friendliness and never-failing courtesy.

V. *New Members* *

The Membership Committee considered the qualifications of 70 nominees. The following 61 nominees recommended by the Committee and the Council were approved for membership at the business meeting:

Altschul, Aaron
Asfour, Raja Y. (C)
Bailey, John M.
Beaton, John R.
Beghin, Ivan D. (C)
Bennett, Mildred S. J.
Brown, Harold (C)
Buskirk, Elsworth
Chalvardjian, Ara M.
Cordano, Angel (C)
Cowan, James W. (C)
Creger, Clarence R.
Csallany, Agnes S.
Danhof, Ivan E.
DiGirolamo, Mario (C)
Donaldson, W. E.
Donovan, Gerald A.
DuPont, Jacqueline
Featherston, W. R.
Fernandez, Nelson (C)
Fleischman, Alan I.
French, Samuel W.
Eades, Charles H.
Goldblith, Samuel
Halsted, James A. (C)
Hemken, Roger W.
Holloszy, John O. (C)
Huber, Agnes M.
Jones, John E. (C)
Kuksis, Arnis
LaChance, Paul A.
Lease, Jane G.
Leatherwood, James M.
Lindenbaum, John (C)
Lopez, Alfredo (C)
Magee, Aden C.
Mueller, Werner J.
McConnell, Kenneth
Morrison, George R. (C)
Neal, Robert A.
Olson, Oscar E.
Orto, Louise A.
Oski, Frank A. (C)
Purser, Douglas B.
Rao, K. K. P. N.
Ritchey, Sanford J.
Robinow, Meinhard
Scheig, Robert (C)
Schneider, Donald
Schwartz, Ruth
Shefer, Sarah
Sinnhuber, Russell
Speckmann, Elwood W.
Stanbury, John B. (C)
Stoewsand, Gilbert
Tannenbaum, Steven
Vivian, Virginia M.
Webb, Ryland E.
Whitaker, JoAnne (C)
Young, Franklin
Young, Vernon R.

* For institutional affiliations and addresses of new members, see the 1967 Federation *Directory of Members.*

(C) Also elected to membership in the Clinical Division at its Annual Meeting, April 29, 1967.

Drs. George H. Beaton, Barbara E. Gunning, Laurence M. Hursh, and Eduardo A. Porta, already members of the American Institute of Nutrition, were elected to membership in the American Society for Clinical Nutrition at its 1967 Annual Meeting.

HONORARY MEMBERS

The following scientists were elected to Honorary Membership in the AIN:

Dr. Joseph Masek, *Director, Institute of Human Nutrition, Prague, Czechoslovakia.* Dr. Masek has been a leader in Czechoslovakia in organizing multi-disciplinary applied nutrition programs and was a key figure in establishing the Society of Rational Nutrition.

Sir Rudolph A. Peters, *Professor Emeritus, Cambridge University, Cambridge, England.* His contributions on the structure of thiamine and its physiological function as cocarboxylase, his broadening concept of "biochemical lesion" as guiding principle in understanding metabolic function of important substances, warrant the high regard in which he is held.

VI. *President's Report*

Sustaining Associate Program. Doctor Schaefer reported on the 1967 Sustaining

Associates which now total 27. (The list of Sustaining Associates is published monthly in the *Journal of Nutrition.*)

Contributions for 1967 ranged from $100 to $1000 for a total of $9,350. Income from this program is used to support in part some of the Institute's activities: expansion of the nutrition career brochure; AIN participation in the development of a dictionary of nutrition terms in collaboration with the NIH; initial support of the establishment of our Office of Nutrition Science Services (see report by O. L. Kline below); travel expenses for foreign participants in AIN symposia; and some assistance in supporting our secretariat.

Career Brochure. Dr. Schaefer advised that the Council had authorized the ad hoc Committee on Career Brochures to (a) improve and expand our present career leaflet, and (b) continue to explore the development of a second, more sophisticated and informative, brochure.

Federation Affairs. The Federation's Life Sciences Research Office has been expanded and renamed the Office of Biomedical Studies. This new Office, supported by government grants, will be headed by Col. T. E. Huber beginning this fall.

The new wing which will complete the Milton O. Lee Building at Beaumont House will be ready for occupancy by mid-year.

The FASEB closed-circuit TV, scheduled for the 1967 meeting, had to be postponed until the 1968 meeting due to programing difficulties. The Federation has received an offer from Telstar to put on a half-hour session on the 1968 meeting. Each society will have equal time to emphasize research aspects of its discipline.

The Federation is establishing a Public Affairs Office to keep in touch with all developments, especially legislative, which might affect the biomedical research community. Each society has been asked to appoint a member for this Committee. The AIN has appointed the Chairman of the new AIN Public Affairs Committee to represent AIN with the Federation Public Affairs Office.

AIN Nutrition Notes. The membership was encouraged to send items of interest to Dr. O. C. Johnson, Editor, AIN Nutrition Notes. Dr. Schaefer stated that it is hoped another issue would be published this year (July).

Council Actions. The Council has established an AIN Public Affairs Committee, and an ad hoc Committee on Biochemical Nutrition to explore how best AIN could recognize and develop the special interests of its "biochemical" members.

The outgoing committee members and representatives were thanked for their efforts on behalf of the AIN, and special thanks were given to Drs. O. L. Kline (outgoing Past President), and N. S. Scrimshaw (outgoing Councilor).

VII. *Secretary's Report*

Dr. W. N. Pearson informed the membership of the following actions taken by the Council in connection with annual meetings.

1. Establishment of an AIN committee to (a) continue evaluation of annual meetings and make recommendations to Council for implementing improvements and (b) to work with the Executive Secretary in programing abstracts for the annual meeting in accordance with the new rules outlined below. Dr. Pearson will be Chairman of this committee.

2. Changes in AIN rules for submission of abstracts.

(a) An AIN member may sponsor (sign) no more than two abstracts and, in compliance with Federation rules, may present orally (as first author) only one of these.

(b) Any sponsored paper not falling within the broad area of nutrition and not accepted as a transfer by an appropriate society shall be returned to the sponsor without being programed or published in the abstracts volume of *Federation Proceedings.*

(c) At the discretion of the Program Committee, any paper that does not lend itself to coherent programing by broad topic may be published but not programed.

(d) Submission of papers dealing primarily with a modification of existing methodology should be discouraged. If submitted, such papers shall be published but not programed. This does not deny presentation of a new exciting advance in methodology.

VIII. *Treasurer's Report*

Dr. W. A. Krehl, Treasurer, discussed briefly the AIN dues breakdown. He pointed out that only $7.00 is received into AIN funds (Clinical Division members pay an additional $2.00 into ASCN funds). The remaining charges cover the cost of subscriptions to one or both of our journals and to *Federation Proceedings*. Although it was the recommendation of the Council that dues not be raised for the 1967–1968 year, the Treasurer advised that the mem-

Balance Sheet, December 31, 1966

ASSETS:		EXHIBIT A
Cash		$ 3,545
Accounts Receivable		16,926
Investments		60,952
Furniture and Equipment	$2,548	
Less accumulated depreciation	(291)	2,257
Prepaid Expenses		132
Total Assets		$83,812
LIABILITIES AND FUND CAPITAL:		
Deferred Income		$ 23
Accounts Payable		7,761
Fund Capital		76,028
Total Liabilities and Fund Capital		$83,812

Statement of Income and Expenditures and Fund Capital
For the Year Ended December 31, 1966

Income:	TOTAL	GENERAL FUNDS	SPECIAL FUNDS (EXHIBIT B)
Membership Dues	$ 6,914	$ 6,914	$ —
Sustaining Associate Memberships	6,000	6,000	—
Annual Meeting Registration	5,291	5,291	—
Journal of Nutrition			
Subscriptions	$ 6,221	$ 6,221	$ —
Editorial Allowance	14,550	14,550	—
Page Charges	22,540	—	22,540
Interest Income	2,319	1,607	712
Grants	28,666	—	28,666
Overhead Income	663	663	—
Miscellaneous Income	327	327	—
Total Income	$93,491	$41,573	$51,918
Expenses:			
Salaries, Payroll Taxes, and Fringe Benefits	$11,483	$ 8,635	$ 2,848
Communications and Travel	6,733	5,491	1,242
Supplies and Duplicating	1,494	1,290	204
Travel Awards	43,366	—	43,366
Journal of Nutrition Editor's Office	10,390	10,390	—
Rent Expense	1,168	1,013	155
Depreciation	209	206	3
Printing and Engraving	724	724	—
Insurance	88	88	—
Miscellaneous Expenses	556	537	19
Total Direct Expenses	$76,211	$28,374	$47,837
FASEB Business Service Charge	1,072	970	102
Total Expenses	$77,283	$29,344	$47,939
Excess of Income over Expenses	$16,208	$12,229	$ 3,979
Fund Capital:			
Balance, December 31, 1965	$59,820	$41,296	$18,524
Balance, December 31, 1966	$76,028	$53,525	$22,503

bership should expect and plan for increasing expenditures in the near future, e.g., to support a full-time Executive Secretary, for expansion of our publication activities, and for development of a more sophisticated and informative career brochure.

The Financial Statement as of December 31, 1966, reproduced here, was presented by Mr. John R. Rice, AIN Business Manager. The AIN Auditing Committee, Drs. W. Gortner and G. V. Vahouny, reported that they had examined the financial report and the records in the AIN business office and found them to be in order.

The recommendation of the Council that the dues remain the same for the 1967–1968 year was approved by the membership.

The Financial Statement was accepted and approved.

IX. *Executive Secretary's Report*

Dr. Waddell reported on the travel grant program in support of nutritionists attending the International Congress of Nutrition (7th), Hamburg, Germany, August 3–10, 1966. Over 190 applications were received and reviewed by the Committee. Funds were more limited than anticipated since grants received from NIH and NSF were less than requested. With the AIN contribution and that of the Nutrition Foundation, Inc., the four sources yielded a total of $44,700. The Committee was able to award 121 travel grants (ranging from $360 to $525, determined by location of traveler). Five of these grants were turned back too late for reassignment. A total of 116 grants was awarded totalling $43,366.

The Office of the Secretariat is now in its third year and, in accordance with part of its purpose, has been taking on an increasing number of functions. As these duties are increased, the position of Executive Secretary more rapidly approaches that of a full-time job. The Council and the Finance Committee are aware of this situation and are studying the monetary income needed to support the acitvities of a full-time Executive Secretary. Dr. Waddell added that the AIN office keeps in close contact with the committees and representatives and will welcome any inquiries from the membership.

X. *Editor's Report — Journal of Nutrition*

Dr. Richard H. Barnes, Editor, Journal of Nutrition, submitted his report for the calendar year 1966. He noted that the time lapse from receipt of manuscripts to publication has increased by one month from 1965 to 1966 (from 5.5 to 6.5), due to publisher's difficulties. The problem should be overcome, however, by the August issue.

The Council has approved an increase in the number of members on the editorial board from 16 to 20. (See end of Proceedings for current board appointments.)

The report and proposed budget presented by the Editor were approved and the report is summarized on page 513.

XI. *Report of the Clinical Division*

Dr. A. B. Eisenstein, Secretary-Treasurer of the ASCN, extended an invitation to AIN members to attend the ASCN Annual Meeting in Atlantic City on April 29. This year the program has been expanded to a full day, with a symposium in the morning and short papers in the afternoon. Dr. Eisenstein announced that the 1967 recipient of the McCollum Award is Dr. Ancel Keys, and stated that all AIN members would be welcome at the Third McCollum Award Banquet on the evening of April 29.

ASCN membership is growing. There was slightly more than a 10% increase in membership in 1966, and based on current action taken by the ASCN Membership Committee, there will be a similar increase in 1967.

The transfer of ownership of the American Journal of Clinical Nutrition from the Reuben H. Donnelley Corporation to the ASCN has been smooth and efficient. The Federation is providing business, subscription, and redactorial services to the ASCN for this Journal. Dr. Eisenstein announced that Dr. Milton Rubini (Chief, Metabolic Section, VA Center, Los Angeles, California) has been appointed as the new Editor of the AJCN, replacing Dr. W. A. Krehl, effective July 1, 1967. From now on all manuscripts for the AJCN should be submitted to Dr. Rubini.

Editing and Publication Operations (Calendar year)

	1964	1965	1966
Volumes published	82, 83, 84	85, 86, 87	88, 89, 90
Pages published (including papers, biographies, announcements and proceedings)	1339	1384	1447
(Scientific papers only)	1267	1313	1350
Papers published (including 3 biographies)	191	189	194
Papers submitted	293	303	316
Papers rejected (rate based on no. papers submitted)	38%	33%	28%
Supplements published	—	—	—
Letters to the Editor	1	2	—
Operating schedule:			
Avg no. days manuscripts with reviewers	20.7	21.5	22.9
Avg no. days manuscripts out for revision	22.6	22.2	24.5
Avg no. days manuscripts in office, in mail or in unavoidable delay	29.2	30.6	30.9
Avg no. days from date of receipt to mailing manuscripts to Wistar	72.5	74.3	78.3
Avg no. months manuscripts with Editorial Office	2.4	2.5	2.6
Avg no. months manuscripts with Wistar Press	3.2	3.0	3.9
Avg no. months from date of receipt to mailing of Journal	5.6	5.5	6.5

Summary of Finances in the Operation of the Editor's Office, *Journal of Nutrition*
January 1, 1966 — December 31, 1966

Balance brought forward	$ 2,207.98
Receipts, AIN	9,400.00
Total receipts and balance available	11,607.98
Expenditures	10,383.21
Balance	$ 1,224.77

The Council approved the budget proposed by Dr. Barnes for the Editor's Office for the year starting January 1, 1967.

XII. *Reports — Committees and Representatives*

A. *Ad hoc Committee on Evaluation of Annual Meetings*: Carl Douglass, Chairman.

Of the 550 members who returned questionnaires, 334 indicated they had attended the 1966 meeting. Of these, 306 rated the short papers as "good" or "average." Despite this, 214 indicated that they would favor some restriction on the number of short papers on the basis of scientific merit. There was strong sentiment on scheduling symposia at times to avoid conflict with short paper sessions. The 1967 program reflected the sentiment of the members.

The recommendations of the Committee included the following: (1) that the number of papers presented at annual meetings be limited to those of genuine scientific merit; (2) that the scientific sessions of the AIN be scheduled so as to reduce the conflicts between short-paper sessions and symposia; (3) that in arranging schedules for meetings, care be exercised not to decrease the time available for informal scientist-to-scientist contacts, and any opportunity to increase time available for this activity should be taken advantage of; (4) that the business meeting should be streamlined by shortening the discussion of routine financial and business matters, and consideration should be given to the possibility of distributing agenda, reports, and proposed resolutions in printed form prior to the meeting, and shortening the time spent on necrologies; and (5) that the banquet should be altered in format so that the introductions and addresses are significantly shortened and their number reduced.

The format for the banquet was altered for 1967 by reducing the number of individuals to be introduced and integrating introductions into the awards ceremony.

The ad hoc Committee on Evaluation of Annual Meetings, having concluded its charge with excellence, was discharged with thanks.

B. *Second Western Hemisphere Nutrition Congress Planning Committee*: W. N. Pearson, Chairman.

A second congress is planned for the week of August 26, 1968 in San Juan, Puerto Rico. Participating organizations are the AIN, the Nutrition Society of Canada, the Latin American Nutrition Society, and the American Medical Association. All participating organizations have representation on the planning committee.

The meeting will last four days and the format will be mostly symposia with some provision for presentation of original communications. A tentative program, with a balance between basic and applied research, has been drawn up.

C. *Publications Management Committee*: W. J. Darby, Chairman.

Dr. Darby advised that the acquisition last summer of the AJCN was the first step in a long-continued effort on the part of AIN to obtain ownership of both official journals. The Publications Management Committee has initiated negotiations with The Wistar Institute to acquire ownership of *The Journal of Nutrition*. The AIN Council has instructed this Committee to continue negotiations with the hope of clarification of transfer by June 30, 1967. Our request to Wistar has been referred to its governing board which will report back to AIN after their May meeting.

D. *Nutrition Science Services*: O. L. Kline, Director.

The proposed project, "Scientific Roster and Related Informational Services in Nutrition for the Agency for International Development, Department of State," for the establishment of services to make available to AID the expertise of the AIN, was submitted to AID by the AIN Committee on International Nutrition in July, 1966. Early in October, the AID agreed in principle to support it. The AIN Council took action at its fall meeting to authorize funds to establish the office on November 1 and the final contract was signed effective December 1 between AID (through the Office of International Research - NIH) and the FASEB. The project resides in the Office of the AIN Secretariat in Beaumont House, Bethesda, Maryland.

Dr. Kline outlined the objectives as follows: (1) The Nutrition Science Services Office acquired the 1966 returns from the National Science Foundation (national register) on IBM punch cards. This information, supplemented by returns on the questionnaire recently sent to AIN members, will be used in the development of a roster of nutrition scientists, with a listing of the interest and experience of each in international nutrition programs. (2) The Institute of International Education, which has a record of all foreign students training in this country, has made available to us its records from September 1960 through June 1965 (about 2,700 students). This information will be used in the development of a locator file of foreign graduate students in nutrition and related fields. (3) In collaboration with appropriate granting agencies, information from published literature, from the Science Information Exchange, from government agency project files, and, to the extent possible, from project lists obtained by polling AIN members, a listing will be compiled of research projects in the U. S. with particular attention to those that relate to nutrition problems in underdeveloped countries. (4) A special sub-committee, of the Project Advisory Committee, has been charged with developing a list of developing-country laboratories and libraries to which subscriptions to selected nutrition publications will be provided for a period of three years, with the hope that after this period they will be able to continue such subscriptions at their own expense. (5) Development of a library of lay-pamphlets and other visual aids useful in public health and nutrition education programs in developing countries.

Dr. Kline thanked the membership for the excellent response to the questionnaire sent recently.

E. *Report on the International Biological Program*: George K. Davis.

During a meeting in Paris last March, a Nutrition Intersectional Working Group meeting was held; Human Adaptability, Use and Management, Marine and Terrestrial Production were represented.

The governing body of the IBP adopted the policy that nutrition should be a separate intersectional program. The working group adopted a number of areas they felt to be of particular importance to be emphasized in the IBP. (1) Nutritional evaluation and acceptability of new foods, foods new to a given population as well as unique products not heretofore used as foods. (2) Evaluation of the nutritive status of populations in contrasting environments, such as high altitudes, at sea level, in the tropics or in arctic areas. (3) Evaluation of nutritional status of isolated populations with emphasis on groups existing on levels of nutrition that differ from our recommended dietary allowances.

The Use and Management group felt that they should lay emphasis on animal and human nutrition and disease (iodine deficiency, selenium); contamination of foods (aflatoxins); nutrition balance (minerals, amino acids); evaluation of NRC requirements; use of wild and tame herbivores (nutrition of these animals and possible source of food); and novel food sources (microbiological, fermentation products).

In the U. S., the U. S. National Committee, IUNS was asked to take leadership in the IBP. The U. S. National Committee for IBP recommended to the Interdepartmental Committee and to the National Academy of Sciences that all departments of the government (such as HEW, USDA, Department of the Interior) include in their budgets a line item (or identify funds) for the IBP. Action is in progress.

The National Science Foundation has informally identified one million dollars for the IBP. We, as nutritionists, must submit specifically identified nutrition projects to apply for such funds.

F. *Report on the International Union of Nutritional Sciences*: C. Glen King, President IUNS.

Dr. King reported on the actions taken during the Hamburg meeting last August. The IUNS has made extensive revisions in their bylaws. The revisions provide for a reorganization of structure to provide for a President and two Vice-Presidents with a council-type management with the officers and six councilors acting as the managing body. Provision has been made for rotation of officers and councilors. A definition of membership in the Union has been outlined; membership must

be on a National basis. Other large or voluntary groups are welcome to be represented at meetings for information only and will have no voting powers. The new bylaws provide for one, two, or three voting delegates from member societies; and the corresponding annual payments to IUNS, initially, will be 25, 75 or 250 pounds sterling. Voting power is increased primarily by the size of membership rather than financial powers. Dr. King advised that the National Academy of Sciences will pay the AIN membership in IUNS beginning January 1, 1967 and will financially aid the working commissions but cannot meet more than a small fraction of the total need.

The President was authorized to appoint, with Council approval, commissions to cover broad areas and working committees to meet specific needs, among which are: Commission on nomenclature, procedures and standards (A. Frazer); Commission on operational programs, such as the IBP; Commission on human development, with special reference to the pre-school child (P. György); Commission on genetic patterns of special nutritional importance (diabetes, metabolic diseases, differentiation of anemias); Commission on nutrition education in schools of medicine, public health, dentistry, veterinary medicine, food science and technology, home economics and dietetics, and agriculture (C. den Hartog); Committee on publications (S. K. Kon), including representation in the management of an official journal of the IUNS and other publications. Dr. King advised that the IUNS is actively exploring an international journal and Dr. R. H. Barnes is the AIN representative to the latter committee.

The National Academy of Sciences has been requested to ask NIH for a contract to support the establishment of a part-time Secretary for the U. S. National Committee of the IUNS. This action is scheduled for the immediate future.

The next International Congress of Nutrition (8th) is scheduled to be held in Prague, Czechoslovakia during the summer of 1969 (August 28–September 5). Professor J. Masek will be the President of the Congress and his associate Dr. Zdenka Slabochova will be General Secretary.

G. *Report of the Public Information Committee*: M. S. Read, Chairman.

Early in the current year the Committee explored a number of activities which might be undertaken by it. Following the discussion of these proposed activities with AIN Council last October, the following will serve to summarize the Committee's accomplishments. (1) The Committee reviewed all abstracts of papers to be presented on the AIN program at the annual meeting in April. Approximately ten per cent of these were recommended to the FASEB Public Information Director for newsworthiness. Specific suggestions on the scientific interest were made. A similar activity will be developed for the ASCN meetings in May, working closely with the AIN Executive Secretary. (2) The Committee has prepared releases for the scientific and lay press recognizing the 1967 Award winners and new Fellows. These were processed through the AIN office with the assistance of the FASEB Public Information Director. (3) The review of manuscripts appearing in the AJCN and JN for their newsworthiness was postponed until early fall at the request of the editors of the journals. (4) Committee members provided suggestions for news items for AIN Nutrition Notes. This procedure needs to be improved for future issues.

H. *Report of the AIN Committee on Experimental Animal Nutrition*: G. F. Combs, Chairman.

Progress has been made during the year in assembling slides depicting nutritional deficiency diseases in experimental animals. Approximately 800 slides have been assembled to date. The Committee's efforts will now turn to the problem of obtaining funds for travel of Committee members to review these slides and the selection of suitable sets of material. Funds will be required also for initial duplication of sets. Details of these needs will be brought to the Council in the future.

Two Conferences were organized for the annual meeting as follows: (1) 32nd Annual Poultry Nutrition Conference, chaired by Hans Fisher. This Conference dealt with "Nutrient requirements for avian reproduction" and current research on fat-soluble vitamins and unidentified growth factors in poultry nutrition. (2) 8th Annual Ruminant Nutrition Conference, chaired by D. S. Kronfeld. The topic was "Nutritional and endocrine influences on calcium metabolism."

I. *International Nutrition Program*

1. *Report of the AIN Committee on International Nutrition*: R. W. Engel, Chairman.

When the AIN Secretariat was established at Beaumont House in January, 1965, one of the important functions envisioned was that of being of service in nutrition science matters to government agencies. This Committee, during the past year, based on earlier recommendations, developed a proposed project to support an Office of Nutrition Science Services in AIN. As a result, a contract has been completed between the Agency for International Development and the FASEB, through the NIH Office of International Research, Nutrition Section. The project was assigned to AIN for implementation.

The new office at Beaumont House, as part of the AIN Secretariat, was opened on November 1, 1966. With assistance of an Advisory Committee which met on January 3, 1967, a program of development was outlined, consistent with terms of the contract. The agreement is to offer services useful in international nutrition programs, particularly for AID. Committee decisions were made regarding the pilot countries to receive immediate attention under the USAID program of "War on Hunger."

Dr. O. L. Kline is the Director of the Office of Nutrition Science Services and activities have already been initiated with respect to the work areas (see report by Dr. Kline, Item XII, D, of these Proceedings).

2. The AIN Council thanked the Committee on International Nutrition for accomplishing its task with excellence and the Committee was dis-

charged. An Advisory Committee to the Office of Nutrition Science Services has been appointed.

J. *Report of the Committee on Nomenclature*: S. R. Ames, Chairman.

1. Organizational. The 1966–1967 Committee on Nomenclature was activated by President Schaefer following the 1966 AIN meeting in Atlantic City with Drs. P. L. Harris, H. H. Williams, H. E. Sauberlich and M. E. Shils as members. The IUNS has established a Commission on Nomenclature. Professor H. Dam (Denmark) is Chairman with Dr. C. G. King and Dr. S. R. Ames as representatives from the U. S. The NAS-NRC Office of Biochemical Nomenclature, Dr. Waldo Cohn, Director, has been most cooperative in continuing to serve as liaison between IUPAC-IUB and this Committee.

2. Vitamin Nomenclature. a) Vitamin E. A system of nomenclature for the stereoisomers of tocopherols and their esters was proposed last year by this Committee, accepted by the AIN Council and published in the Proceedings (J. Nutr., *90:* 108–109, 1966). This nomenclature system was submitted to IUPAC-IUB Commission on Biochemical Nomenclature and designated CBN 1965-7. Consideration of this system was referred to a sub-committee of CBN on Carotenoid Nomenclature which met at Trondheim in June, 1966. There has been correspondence on this system but tentative rules have not yet appeared. Further action by this Committee will depend on the response from the CBN Committee. b) Vitamins. IUPAC-IUB-CBN has revised the rules on vitamin nomenclature appearing in J. Amer. Chem. Soc., 82: 5581–5583, 1960, and the following rules were published in J. Biol. Chem., *341:* 2987, 1966: Trivial Names of Miscellaneous Compounds of Importance in Biochemistry; Nomenclature of Quinones with Isoprenoid Side Chains; Nomenclature and Symbols for Folic Acid and Related Compounds; and Rules for the Nomenclature of Corrinoids.

Professor H. Dam and Dr. T. Moore as representatives of IUNS took exception to the above published rules and submitted a detailed report on their views at the International Congress of Nutrition (7th) at Hamburg on August 10, 1966. A cablegram was dispatched to Dr. P. L. Harris of this Committee for presentation to the Congress endorsing their suggested program of action and requesting representation for AIN on the proposed IUNS permanent commission.

Subsequently, the IUNS Council decided to establish a Commission on Nomenclature within IUNS. Professor H. Dam was designated Chairman and Dr. Dorothy Duncan, Secretary. Representatives from the U. S. are Drs. S. R. Ames and C. G. King. This Commission has requested comments and criticisms of the Dam-Moore report as well as suggestions on other nomenclature problems. Detailed comments on the Dam-Moore report will be submitted following the meeting of this Committee in Chicago in April, 1967. In the meantime, the Editors of the British Journal of Nutrition and of the Proceedings of the Nutrition Society have adopted the earlier IUPAC-IUB-CBN nomenclature with certain exceptions (Brit. J. Nutr., 20 *(no. 1):* (v), 1966, and Proc. Nutr. Soc., 25 *(no. 1):* (v)–(vi), 1966, Directions to Contributors). They retain the name "vitamin" for vitamins A, D, E, K, B_1, B_6, B_{12}, and C to cover the biological activity when more than one active substance is involved. Unfortunately, their summary appears to be based on the earlier version of the IUPAC-CBN nomenclature (Journal of the American Chemists' Society, 1960), and does not include the rules published recently (Journal of Biological Chemistry, 1966). It is recommended that no action should be taken by this Committee or by AIN until consideration can be given to the recommendations of the IUNS Commission on Nomenclature.

3. Other Activities. In a letter to the Food and Nutrition Board, the International Organization for Standardization advocated adoption of either "joule" or "kilowatt hour" as a unit of heat (energy) in preference to "calorie" or "kilocalorie." Application of this proposal to the science of nutrition would be very difficult. The problem is currently under consideration by this Committee.

4. Chairman's Comments and Recommendations. In contrast with the previous lack of activity on nomenclature problems in nutritional science, there has been an encouraging upsurge of interest at the international level. It is recommended that the Committee on Nomenclature be continued for 1967–1968, that it continue to cooperate closely with all international groups, and that action on vitamin nomenclature be temporarily postponed pending review by the IUNS Commission on Nomenclature.

Summary. A system of nomenclature for the stereoisomers of the tocopherols was accepted by the Council, published in the Journal of Nutrition and submitted to IUPAC-IUB-CBN for their consideration.

Continued close liaison with IUNS was insured with appointment of Drs. King and Ames to the newly formed IUNS Commission on Nomenclature. Action on Vitamin Nomenclature is temporarily postponed pending recommendations of the IUNS Commission.

K. *Report of the AIN Representative to the AAAS*: Ruth M. Leverton.

The Committee on Council Affairs had been requested to consider means by which the AAAS Council could achieve a membership that would be more accurately representative of the different fields of scientific interest than is now found in the Council. This will be studied in 1967.

The Study Committee on Cooperation with Developing Countries recommended that a full-time Office of International Science be established within the Association and suggested a number of activities considered appropriate for the proposed office. Because a number of questions of cost and program, as well as desirability, and relations with other bodies undertaking such work needed to be considered, the Committee on Council Affairs recommended that action on the recommendation be deferred for another year to give the staff and Board of Directors an opportunity to study the proposal.

Extensive discussion centered on a proposed resolution regarding the AAAS position on the

uses of biological and chemical agents to modify the environment. The resolution as it was adopted reads as follows:

> WHEREAS modern science and technology now give man unprecedented power to alter his environment and affect the ecological balance of this planet; and
> WHEREAS the full impact of the uses of biological and chemical agents to modify the environment, whether for peaceful or military purposes, is not fully known;
> BE IT RESOLVED that the American Association for the Advancement of Science: (1) expresses its concern regarding the long-range consequences of the use of biological and chemical agents which modify the environment; and (2) establishes a committee to study such use, including the effects of chemical and biological warfare agents, and periodically to report its findings through appropriate channels of the Association; and (3) volunteers its cooperation with public agencies and offices of government for the task of ascertaining scientifically and objectively the full implications of major programs and activities which modify the environment and affect the ecological balance on a large scale.

A panel discussion followed including the subject: "Who should be responsible for planning science policy: PSAC, the National Science Board, others?"; "Distribution of research funds to different areas of science"; "New sources for ideas on science policy"; and "Possible models for flow planning of scientific manpower in anticipation of reductions in the rate of increase of funds for research."

XIII. *Future Annual Meetings*

1968: Atlantic City, New Jersey, April 16–20, registration opens April 15
1969: Atlantic City, New Jersey, April 14–18, registration opens April 13
1970: Atlantic City, New Jersey, April 13–17, registration opens April 12
1971: Chicago, Illinois, April 13–17, registration opens April 12

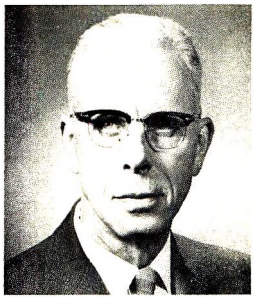
ANNUAL DINNER AND PRESENTATION OF FELLOWS AND AWARDS

The annual banquet was held on Wednesday, April 19, 1967 at the Sheraton-Blackstone Hotel with 402 attending. Dr. Schaefer presided.

Dr. Sam Hansard introduced the newly appointed Fellows, whose citations follow:

GEORGIAN ADAMS

GEORGIAN ADAMS — for an illustrious career as liaison officer in the federal service (USDA); for contributions to methodology and to basic knowledge of the composition and nutritive value of foods; for creatively fostering research in broad areas of human nutrition and biochemistry and the promotion of sound research programs of local, regional, national, and international scope; for unusual competency in administrative implementation of cooperative research programs. In her professional capacity she has influenced the careers and research accomplishments of many AIN members. Dr. Adams' accomplishments have been recognized by the Superior Service Award of the USDA and by an honorary Doctor of Science degree from her alma mater, the University of Nebraska.



EARLE W. CRAMPTON

EARLE W. CRAMPTON — for a distinguished career of research in both fundamental and applied nutrition, particularly in the development of swine feeding programs, in the nutritional evaluation of forages and other feeds, and in the application of appropriate experimental designs and statistical methods to animal experiments. As a teacher, he has influenced a large number of undergraduates to embark upon graduate studies; as a graduate research director, he has moulded the careers of many nutritionists through his personal devotion to work, his ability to perceive, pursue and critically evaluate nutritional problems, and his unrelenting insistence upon excellence of performance. As a member of scientific and professional societies, as well as of national committees and councils, he has contributed to the advancement of the profession of nutrition in both Canada and the United States.

L. EMMETT HOLT, JR.

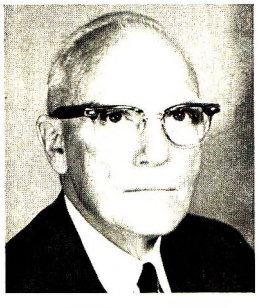
L. EMMETT HOLT, JR. — for a brilliant and highly productive career in experimental nutrition as well as in practical pediatrics; particularly for his contributions to the mechanism of calcification in animal tissues; the comparative value of different fats in infant nutrition; the requirements for B vitamins in adults and children; and, above all, for the thoroughgoing study of the amino acid and protein requirements of infants and children. His group identified the chemical basis of an anomaly of amino acid metabolism, maple syrup urine disease, and demonstrated its control by diet. He has received many awards recognizing his accomplishments including the 1964 AIN Osborne and Mendel Award.

AIN MEAD JOHNSON AWARD FOR RESEARCH IN NUTRITION

WILLIAM N. PEARSON

The 1967 Mead Johnson and Company Award of $1,000 and a scroll was awarded to Dr. William N. Pearson, Associate Professor of Biochemistry, Vanderbilt University. It was presented in consideration of his fundamental contributions to our understanding of the metabolism of thiamine; for his basic studies on iron and selenium metabolism; for his contributions to the field of biochemical appraisal of nutritional status; and for his continuing contributions and activities in the field of international nutrition.

BORDEN AWARD IN NUTRITION



RICHARD H. BARNES

The American Institute of Nutrition's 1967 Borden Award of $1,000 and a gold medal was presented to Dr. Richard H. Barnes, Professor of Nutrition and Dean of the Graduate School of Nutrition, Cornell University, Ithaca, New York. The award was given for his research on protein malnutrition in experimental animals, involving low-protein diets such as cause kwashiorkor in humans, studies on the value of milk and other dietary proteins in counteracting protein malnutrition, and the effect of protein deficiency on the mental development of the young; for research on the factors in raw soybeans that impair the utilization of soybean proteins; and the role of coprophagy in the nutrition of the rat in which was shown great ingenuity, persistence and experimental skill.

OSBORNE AND MENDEL AWARD

SAMUEL LEPKOVSKY

The 1967 Osborne and Mendel Award of $1,000 and a scroll was presented to Dr. Samuel Lepkovsky, Professor of Poultry Husbandry, University of California, Berkeley. It was given in recognition of his outstanding studies on physiological mechanisms as they relate to food intake and to stress. His work has opened new vistas on the effect of the hypothalamus (through the central nervous system) and various endocrine systems on appetite, satiety and nutritional processes. Using the chick and the rat, he has developed new techniques which are useful in understanding the biological mechanisms dealing with the acceptance and utilization of new food products.

THE CONRAD A. ELVEHJEM AWARD FOR PUBLIC SERVICE IN NUTRITION

JOHN E. YOUMANS

The 1967 Conrad A. Elvehjem Award of $1,000 and a scroll was presented to Dr. John B. Youmans, Vice-President of the Grayson Foundation and President Emeritus of the United Health Foundations, Inc. The award was made in recognition of Dr. Youmans' pioneering efforts in public health nutrition during a long and distinguished career as physician, medical educator, military scientist, and public servant; also for his contributions to nutritional science, and his vigorous support of the importance of nutrition in medical practice, in military planning, and in public health programs in the United States and in other countries.

AMERICAN INSTITUTE OF NUTRITION

Founded September 27, 1928; Incorporated November 16, 1934; Member of Federation 1940

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Ad hoc Committee AIN National Medal of Science Committee: W. N. Pearson, chairman; R. W. Engel, R. E. Shank.

Ad hoc Planning Committee for Second Western Hemisphere Nutrition Congress: W. N. Pearson, chairman; Conrado Asenjo, J. E. Dutra de Oliveira, A. B. Eisenstein, L. E. Lloyd, P. L. White.

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(A Division of the American Institute of Nutrition)

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American Journal of Clinical Nutrition

M. E. Rubini, Editor-in-Chief; R. E. Hodges, Associate Editor; Margaret J. Albrink, Ernest Beutler, G. J. Gabuzda, Francisco Grande, S. A. Hashim, Victor Herbert, Robert Jackson, C. M. Leevy, M. E. Shils, D. M. Watkin, George M. Owen, H. E. Sauberlich.

Invitation for Nominations for 1968 American Institute of Nutrition Awards

Nominations are requested for the 1968 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) *A brief convincing statement setting forth the basis for the nomination and, where appropriate, a selected bibliography which supports the nomination. Seconding or supporting letters are not to be submitted.* (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1967*, to be considered for the 1968 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age except as specified for the Mead Johnson Award. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award. A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person, but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made during the banquet at the annual meeting.

1968 Borden Award in Nutrition

The Borden Award in Nutrition, consisting of $1000 and a gold medal, is made available by the Borden Company Foundation, Inc. The award is given in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of milk or its components. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. Employees of the Borden Company are not eligible for this award nor are individuals who have received a Borden Award from another administering association unless the new award be for outstanding research on a different subject or for specific accomplishment subsequent to the first award.

Former recipients of this award are:

1944 – E. V. McCollum
1945 – H. H. Mitchell
1946 – P. C. Jeans and Genevieve Stearns
1947 – L. A. Maynard
1948 – C. A. Cary
1949 – H. J. Deuel, Jr.
1950 – H. C. Sherman
1951 – P. György
1952 – M. Kleiber
1953 – H. H. Williams
1954 – A. F. Morgan and A. H. Smith
1955 – A. G. Hogan
1956 – F. M. Strong
1957 – no award
1958 – L. D. Wright
1959 – H. Steenbock
1960 – R. G. Hansen
1961 – K. Schwarz
1962 – H. A. Barker
1963 – Arthur L. Black
1964 – G. K. Davis
1965 – A. E. Harper
1966 – R. T. Holman
1967 – R. H. Barnes

NOMINATING COMMITTEE:

P. H. WESWIG, *Chairman*
K. E. HARSHBARGER
R. T. HOLMAN

Send nominations to:

DR. P. H. WESWIG
Department of Agricultural Chemistry
Oregon State University
Corvallis, Oregon 97331

1968 Osborne and Mendel Award

The Osborne and Mendel Award of $1000 and an inscribed scroll has been established by the Nutrition Foundation, Inc., for the recognition of outstanding recent basic research accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the

most significant published contribution in approximately the calendar year preceding the annual meeting of the Institute, or who has published recently a series of papers of outstanding significance. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration.

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
1957 – G. R. Cowgill
1958 – P. György
1959 – Grace A. Goldsmith
1960 – N. S. Scrimshaw
1961 – Max K. Horwitt
1962 – William J. Darby
1963 – James B. Allison
1964 – L. Emmett Holt, Jr.
1965 – D. M. Hegsted
1966 – H. H. Mitchell
1967 – Samuel Lepkovsky

NOMINATING COMMITTEE:

ALEX BLACK, *Chairman*
L. E. HOLT, JR.
M. K. HORWITT

Send nominations to:

DR. ALEX BLACK
Pennsylvania State University
Agricultural Experiment Station
University Park, Pennsylvania 16802

1968 Mead Johnson Award for Research in Nutrition

The Mead Johnson Award of $1000 and an inscribed scroll is made available by Mead Johnson and Company to an investigator who has not reached his 46th birthday during the calendar year in which the Award is given. Selection by the Jury of Award will be based primarily on a single outstanding piece of research in nutrition published in the year preceding the annual meeting or on a series of papers on the same subject published within not more than the three years preceding the annual meeting.

Former recipients of this award are:

1939 – C. A. Elvehjem
1940 – W. H. Sebrell, Jr.
J. C. Keresztesy
J. R. Stevens
S. A. Harris
E. T. Stiller
K. Folkers
1941 – R. J. Williams
1942 – G. R. Cowgill
1943 – V. du Vigneaud
1944 – A. G. Hegan
1945 – D. W. Woolley
1946 – E. E. Snell
1947 – W. J. Darby
P. L. Day
E. L. R. Stokstad
1948 – F. Lipmann
1949 – Mary S. Shorb
K. Folkers
1950 – W. B. Castle
1951 – no award
1952 – H. E. Sauberlich
1964 – J. S. Dinning
1965 – J. G. Bieri
1966 – M. Daniel Lane
1967 – W. N. Pearson

NOMINATING COMMITTEE:

A. R. KEMMERER, *Chairman*
L. M. HENDERSON
H. E. SAUBERLICH

Send nominations to:

DR. A. R. KEMMERER
University of Arizona
Agricultural Sciences Building
Tucson, Arizona 85721

1968 Conrad A. Elvehjem Award for Public Service in Nutrition

The Conrad A. Elvehjem Award for Public Service in Nutrition, consisting of $1000 and an inscribed scroll, is made available by the Wisconsin Alumni Research Foundation. The award is bestowed in recognition of distinguished service to the public through the science of nutrition. Such service, primarily, would be through distinctive activities in the public interest in governmental, industrial, private, or international institutions but would not exclude, necessarily, contributions of an investigative character.

Former recipients of this award are:

1966 – C. Glen King
1967 – J. B. Youmans

NOMINATING COMMITTEE:

F. W. QUACKENBUSH, *Chairman*
R. E. SHANK
OLAF MICKELSEN

Send nominations to:

DR. FORREST W. QUACKENBUSH
Department of Biochemistry
Purdue University
Lafayette, Indiana 47907

Invitation for Nominations for 1968 American Institute of Nutrition Fellows

The Fellows Committee of the American Institute of Nutrition invites nominations for Fellows in the Society. Eligible candidates are active or retired members of the Society who have passed their sixty-fifth birthday (by the time of the annual meeting) and who have had distinguished careers in nutrition. Up to three Fellows may be chosen each year.

Nominations may be made to the Chairman of the Fellows Committee by any member of the Society, including members of the Committee.

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable.

Final selection will be made by the Fellows Committee and a suitable citation will be presented at the Annual Dinner in April.

Fellows Committee:

W. H. GRIFFITH, *Chairman*
AGNES F. MORGAN
RICHARD M. FORBES
T. H. JUKES
L. A. MAYNARD

Send nominations to:

DR. W. H. GRIFFITH
Federation of American Societies for Experimental Biology
9650 Rockville Pike
Bethesda, Maryland 20014

The following persons have been elected previously as Fellows of the Society:

Georgian Adams (1967)
J. B. Brown (1964)
Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Earle W. Crampton (1967)
Henrik Dam (1964)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Wendell H. Griffith (1963)
Paul György (1965)
Albert G. Hogan (1959)
L. Emmett Holt, Jr. (1967)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
J. S. Hughes (1962)
C. Glen King (1963)
Max Kleiber (1966)
Samuel Lepkovsky (1966)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
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Leo C. Norris (1963)
Helen T. Parsons (1961)
Lydia J. Roberts (1962)
William C. Rose (1959)
W. D. Salmon (1962)
Arthur H. Smith (1961)
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Harry Steenbock (1958)
Hazel K. Stiebeling (1964)
Raymond W. Swift (1965)
Robert R. Williams (1958)
John B. Youmans (1966)

Invitation for Nominations for Honorary Membership in the American Institute of Nutrition

The Committee on Honorary Memberships of the American Institute of Nutrition invites nominations for Honorary Members.

Distinguished individuals of any country who are not members of the American Institute of Nutrition and who have contributed to the advance of the science of nutrition shall be eligible for proposal as Honorary Members of the Society.

Nominations may be made to the Chairman of the Committee on Honorary Memberships by two members of the Society.

Nominations (in three copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

Final selection of nominees will be made by the Council of the American Institute of Nutrition and such nominations submitted to the Society at the spring meeting. Election requires a two-thirds majority of the ballots cast.

Honorary members pay no membership fees but are eligible to subscribe to the official journal(s) at member's rates.

Committee on Honorary Memberships:

GRACE GOLDSMITH, *Chairman*
R. W. ENGEL
L. C. NORRIS

Send nominations to:

DR. GRACE GOLDSMITH
Tulane University School of Medicine
New Orleans, Louisiana 70112

The following persons have been elected previously as Honorary Members of the Society:

Kunitaro Arimoto
W. R. Aykroyd
Frank B. Berry
Edward Jean Bigwood
Frank G. Boudreau
Robert C. Burgess
Dame Harriette Chick
F. W. A. Clements
Sir David P. Cuthbertson
Herbert M. Evans
Joachim Kühnau
Joseph Masek
Toshio Oiso
H. A. P. C. Oomen
Lord John Boyd Orr
Conrado R. Pascual
V. N. Patwardhan
Sir Rudolph A. Peters
B. S. Platt
Emile F. Terroine
Jean Tremolieres
Eric John Underwood
Artturi I. Virtanen

Invitation for Nominations for the 1968 Hoblitzelle National Award in the *Agricultural Sciences*

The Hoblitzelle Award Committee of the American Institute of Nutrition invites nominations for the 1968 Hoblitzelle National Award in the Agricultural Sciences.

The Hoblitzelle Award, consisting of $10,000, a gold medal and an attesting certificate, is presented in recognition of the outstanding contribution in the agricultural sciences which has been published during the preceding four-year period. The donor of the Award is the Hoblitzelle Foundation of Dallas, Texas. The Award is administered by the Texas Research Foundation, Renner, Texas.

All American scientists working in the United States and its territories, irrespective of creed, color, nationality, sex, age, branch of science, or affiliation with scientific or scholastic organizations, are eligible. Either an individual or team of scientists may be nominated for the Award, but a team must include only those scientists who have contributed the basic ideas. Preferably no more than two scientists should be nominated as a team.

This Award covers research in agronomy, animal science, bacteriology, biochemistry, botany, entomology, genetics, horticulture, nutrition, soil science, veterinary science, zoology, and such other sciences as may be deemed to serve agriculture in their broadest aspects.

The various professional societies related to the agricultural sciences, the Agricultural Research Service and the Agricultural Experiment Stations have been requested to receive and screen nominations for the Award. Each may forward three nominations to the Texas Research Foundation from which the Final Awards Committee will select three candidates. The Hoblitzelle Foundation will select the recipient, or recipients, from these candidates.

The nomination for the Award must be accompanied by a complete set of publications by the scientist covering his scientific accomplishment, and a comprehensive evaluation of the potential significance of the work. The dates of publications for the 1968 Award must fall within the period January 1, 1963 through December 31, 1966. Final date for submission of nominations is November 15, 1967.

AIN Hoblitzelle Award Committee:

Orville G. Bentley, *Chairman*
R. W. Engel
R. G. Hansen
H. O. Kunkel

Send nominations to:

Dr. Orville G. Bentley
Dean of Agriculture
University of Illinois
Urbana, Illinois 61803

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