Synthesis of Thiamine in the Digestive Tract of the Rat[1,2]

BERNARD S. WOSTMANN AND P. LEONARD KNIGHT[3]
Lobund, Department of Biology, University of Notre Dame, Notre Dame, Indiana

The ability of the intestinal flora of the rat to synthesize thiamine is no longer questioned, but the availability of the flora-synthesized thiamine to the host is still a much debated problem. Abdel-Salaam and Leong ('38) grew cecal bacteria in vitamin B_1-free broth and concluded that the newly formed vitamin remained in the bacterial cell instead of being released into the surrounding medium. *In vivo* studies of cecal contents by Mitchell and Isbell ('42) indicated that there was a greater amount of thiamine in the bacteria than in the medium surrounding the bacteria. They estimated that in their experiments bacterial production of thiamine added 8 to 18% to a dietary intake of approximately 30 μg. Later investigators noted that with diets containing insufficient thiamine to sustain growth, more of the vitamin seemed available to the animal when antibiotics were included in the diet, presumably because of an effect of these compounds on the intestinal flora (Jones and Baumann, '55; Scott and Griffith, '57; Wostmann et al., '58). Mameesh and Johnson[4] administered radiothiamine with the diet and estimated the possible isotope dilution effect in the liver. From their data they concluded that with an adequate diet, little or no flora-synthesized thiamine was taken up by the host. Mameesh et al. ('59) later corroborated by Barnes and his associates ('60), showed that improved growth occurred with a thiamine-limiting diet containing penicillin only when coprophagy was allowed.

The pathway of bacterial synthesis of thiamine has been investigated by many Japanese workers. Shimomura et al. ('57) concluded that synthesis was via the cysteine route while Yamada ('57) suggested that it was via the methionine pathway. Previous observations in which S^{35} as inorganic sulfate was fed to rabbits and sheep (Kulwich et al., '57)[5] indicated that cysteine and methionine were the two major organic compounds in which radiosulfur was incorporated by the bacterial flora. In this paper we report direct evidence that in the intestinal tract of the rat fed a complete diet, thiamine containing S^{35} from $S^{35}O_4^=$ (originally carrier-free H_2SO_4) when administered by stomach tube or cecal injection is synthesized by the microbial flora. Comparison of the thiamine concentrations in the intestinal contents of conventional and germ-free rats indicated the cecum as the major, if not only, site of bacterial synthesis. The flora-synthesized thiamine is not available to the host at the site of synthesis nor en route to its elimination in the feces (first alimentary passage). Furthermore, when coprophagy was allowed (second passage), there was again no evidence, under the conditions of the experiment, that the intestinally produced vitamin was available to the host to any appreciable extent.

Received for publication January 14, 1961.

[1] This research was supported specifically by the Nutrition Foundation, Inc. (Grant no. 257); also generally by the Office of Naval Research and the Army Surgeon General (Contract NONR-1623), by National Institutes of Health (Grant A-566) and by the University of Notre Dame.

[2] A preliminary report of some of this work was presented before the 5th International Nutrition Congress, 1960, Washington, D. C.

[3] St. Mary's College, Notre Dame, Indiana.

[4] Mameesh, M. S., and B. C. Johnson 1958 Effect of penicillin on intestinal synthesis of thiamine in the rat. Federation Proc., *17:* 483 (abstract).

[5] Kulwich, R., L. Struglia, J. T. Jackson and P. B. Pearson 1954 Synthesis of cystine and methionine from S^{35} labeled sodium sulfate in the rabbit. Federation Proc., *13:* 463 (abstract).

EXPERIMENTAL

Lobund strain male and female rats of Wistar origin were used in all experiments. In one series in which the thiamine concentrations in the intestinal contents of germ-free and conventional animals were directly compared, the germ-free rats were taken from the germ-free colony housed in the Reynier's system (Reyniers, '56). The conventional animals in this and other experimental series were taken from the genetically closely related conventional rat colony. Sacrifice, harvesting of samples and the thiamine determination were as described hereafter.

In all other series only conventional rats were used. They were administered $S^{35}O_4^=$ by stomach tube or by injection directly into the cecum. The tracer was injected into the cecum using a tuberculin syringe with a no. 24 needle after a short incision was made in the abdomen. The wound was closed with surgical clips. The dosage of S^{35} varied between 50 and 500 μc in approximately 0.5 ml of 100 mg per 100 ml of Na_2SO_4. During the experimental period, each animal was housed in an individual screen cage (9 × 2 × 2 inches) to prevent coprophagy. Prior to the experiment, all animals had been subjected to this confinement for at least 5 hours on 5 consecutive days to condition them to the experimental environment. Before and during the experiment, complete diets (L-356 or Rockland[6]) and water were available ad libitum. The autoclaved L-356 diet (Larner and Gillespie, '57) was steam-sterilized for 25 minutes at 123°C. It contained 60 mg of thiamine hydrochloride per kg before and 8.8 mg per kg after sterilization. The commercial Rockland diet contained 4.4 mg per kg. The experimental period ranged from three to 48 hours. One 48-hour experiment was conducted in which approximately one-half the animals were allowed coprophagy by normal housing, and in the other half coprophagy was prevented as described above. At the end of the period, the animals were anesthetized with ether and decapitated. The liver, heart, feces and contents of the small intestine, cecum and colon were frozen and stored (−24°C) for later analysis of total thiamine and radiothiamine.

The xiphoid process was also isolated, washed with saline, acetone and ether and dried in an oven at 100°C (average weight, 6.8 mg). Pelc and Glucksmann ('55) found a rapid uptake of S^{35} by the xiphoid process of the mouse after injection of 250 μc of the tracer intraperitoneally. This suggested that the uptake of S^{35} in the xiphoid process of the rat could be used to detect a possible effect of the tracer dose on the rate of absorption from the gastrointestinal tract and thus gauge the normalcy of the tract under the experimental conditions. The observations in table 1 show that absorption apparently was not greatly affected by dosages ranging from 50 to 450 μc. Furthermore the data in table 2 indicate that the highest dose used (500 μc) did not result in any

[6] Rockland Rat Diet, A. E. Staley Manufacturing Company, Decatur, Illinois.

TABLE 1

Radioactivity of xiphoid process after administration of $S^{35}O_4^=$

Total counts administered	Counts/mg/min. at time of sacrifice recalculated to dose = 10^8 counts			
	7 hours		24 hours	
	Stomach tube	Cecal injection	Stomach tube	Cecal injection
$2 \times 10^7 = 50$ μc	142(2)[1]	—[2]	92(2)	—
3.3×10^7	108(2)	88(3)	110(1)	88(3)
$4.0 \times 10^7 = 100$ μc				
6.8×10^7	164(3)	100(2)	—	88(3)
$1.0 \times 10^8 = 250$ μc	—	64(5)	—	46(2)
1.4×10^8	—	61(6)	—	—
$1.8 \times 10^8 = 450$ μc	161(3)	—	99(3)	—

[1] Numbers in parentheses denote total animals used.

[2] — Not determined.

TABLE 2

Comparison of micro-organisms of ileum in untreated rats and rats administered 500 μc $S^{35}O_4^=$ by stomach tube[1]

Micro-organisms	Treated		Untreated	
	Rat no. 1	Rat no. 2	Av. 3 rats	Range
Total count (aerobic)	10.8[2]	9.6	9.6	(8.6–9.8)
Total count (anaerobic)	10.7	9.4	9.8	(8.5–10.2)
Lactobacilli	10.7	9.4	9.8	(8.6–10.2)
Streptococci	9.4	8.4	8.0	(7.7–8.3)
Coliforms	5.6	7.9	5.8	(5.7–5.9)
Yeasts	7.2	7.7	8.3	(7.7–8.7)
Micrococci	3.9	4.3	?	(0–6.1)
Clostridia	0	0	6.4	(4.0–6.9)

[1] Time of sacrifice, 7 hours.

[2] Values expressed as log of numbers per gram dry weight.

major changes in the intestinal flora, except for a possible absence of Clostridia in rats which received this dose.

Thiamine was determined by a modification of the thiochrome method as outlined by Bouman ('48). Bound thiamine was freed by adding 50 mg of papain and 25 mg of takadiastase at pH 4.6 and incubating in a water bath at 45°C for two hours. After precipitation of the proteins with 10% trichloroacetic acid and centrifugation at 1600 rpm, the supernatant was decanted. Twenty-five milliliters of saturated NaCl solution were added to the supernatant and the volume was brought to 80 ml with distilled water. After extraction with isobutanol to remove interfering substances, an aliquot of the aqueous phase was taken for direct chemical determination using a Coleman photofluorometer.

To obtain a correct impression of the radioactivity of the thiamine isolated from the intestinal contents, heart and liver, high purity of the final samples was a first requirement. To achieve this, the remainder of the thiamine-containing aqueous phase was treated as outlined hereafter, but only after 1000 μg of nonradioactive thiamine dihydrochloride[7] had been added to insure a fair recovery.

The remaining solution was passed through a Decalso column, followed by adsorption of thiamine on franconite (a type of fuller's earth) and subsequent elution, oxidation to thiochrome and extraction into isobutanol. The thiochrome from this solution was then absorbed on a second Decalso column, eluted with 10 ml of a 10% NaCl solution adjusted to pH 1, and in this solution selectively precipitated by adding a solution of Reinecke salt in CH_3OH (Bandelin and Tuschoff, '53).

The precipitate was dissolved in a 1:1 (vol/vol) mixture of acetone-isobutanol. Aliquots of the dissolved precipitate were then used as follows: (1) extracted into isobutanol and read in a photofluorometer for a direct determination of the recovery of the original and added thiamine (making possible the calculation of the amount of thiamine which was derived from the original sample and which was present in the aliquot); (2) placed on a filter paper disc in a stainless steel planchet, air dried, and counts taken using a Tracerlab TGC-14 counter; and (3) spotted on Whatman no. 1 filter paper. Ascending chromatography was run using as a solvent 1-propanol/water/acetate buffer pH 5 (70:20:10) (Siliprandi and Siliprandi, '54) in an apparatus described by Huo Ping-Pan ('56). The paper was air-dried and passed through an isotope chromatogram scanner which was connected to a recorder. The speed of the recorder and scanner was 1.5 inches per hour. The peak of radioactivity coincided with the thiochrome spot (fluorescing in ultra-violet light) (fig. 1). Rf values of the unknown (presumably radioactive thiochrome) and of a thiochrome standard[8] under the same conditions were each about 0.68. The thiochrome spot was then cut out of the

[7] Merck and Company, Rahway, New Jersey.

[8] Nutritional Biochemicals Corporation, Cleveland.

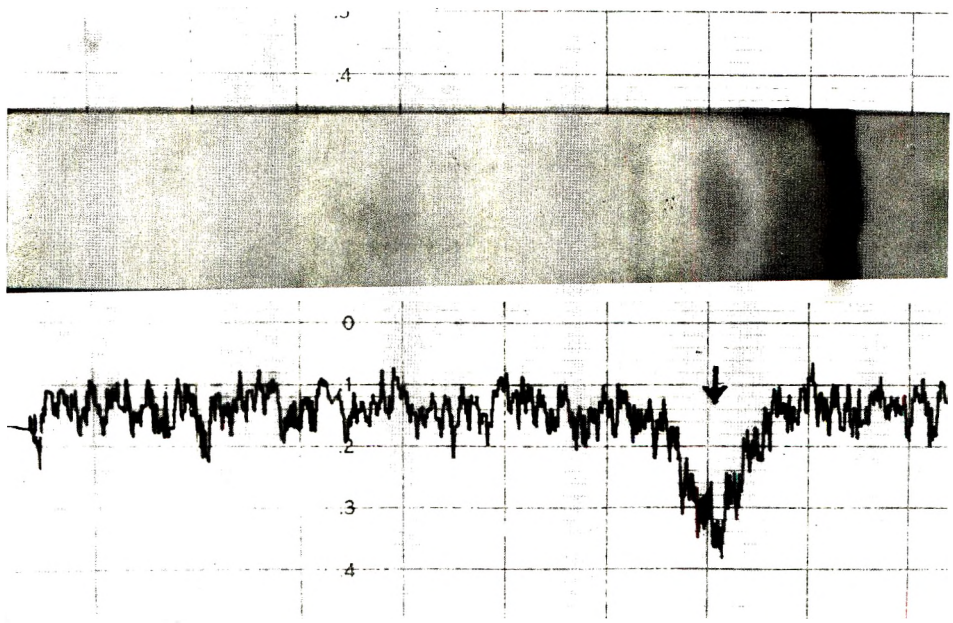

Fig. 1 Chromatogram of thiochrome (isolated by precipitation with Reinecke salt in methyl alcohol; see text) showing the fluorescent spot of thiochrome coinciding with the peak of radioactivity recorded by the chromatogram scanner. Solvent, 1 propanol/water/acetate buffer pH 5 (70:20:10). Speed of recorder and scanner, 1.5 inches/hour.

chromatogram, placed in a planchet and counts taken on it. The counts were approximately one-half the value described previously under (2). Knowing the amount of thiamine originally present, under (1), and the value from (3), the specific activity was calculated and expressed as counts/μg of vitamin B_1/minute.

RESULTS AND DISCUSSION

Many authors refer to the cecum and the colon as the main sites of microbial synthesis of vitamins (Guerrant et al., '34, '35, '37). In table 3 the thiamine concentration in the contents of the digestive tract of conventional and germ-free rats is compared. In the tract of the germ-free animal the amount of vitamin B_1 decreases from the upper part of the intestine to the colon. In the conventional rat, however, the vitamin content decreases in the small intestine but shows a sharp increase in the cecum. These data unquestionably confirm that in the case of thiamine most of the synthesis by the microbial flora occurs in the cecum.

The radioactivity of the thiamine in the various parts of the intestinal tract after radiosulfate administration are shown in tables 4 and 5. This indicates that the intestinal flora produces thiamine with the incorporation of $S^{35}O_4^{=}$, directly or via

TABLE 3

Thiamine in contents of intestinal tract of 100-day-old germ-free and conventional rats fed diet L-356

Type of animal	Intestine, middle third	Intestine, lower third	Cecum	Colon
		μg/gm dry weight		
Germ-free	4.5 ± 0.4[1](6)[2]	2.3 ± 0.1(16)	1.9 ± 0.1(17)	1.7 ± 0.1(19)
Conventional	8.7 ± 0.3(6)	4.5 ± 0.2(13)	8.5 ± 0.4(19)	8.0 ± 0.4(17)

[1] Standard deviation of the mean.
[2] Numbers in parentheses indicate total animals used.

TABLE 4

Specific activity of thiamine (counts/μg/min.) in intestinal contents after administration of $S^{35}O_4^=$ by stomach tube to adult conventional rats[1,2,3]

Dose	Time of sacrifice (hours)	Lower third small intestine	Cecum	Colon	No. of animals
μc					
200	7	+[4]	79 ± 12[5]	—[6]	4
400	24	0	52 ± 6	—	5
4 × 200/12 hours	48	approx. 130	315 ± 12	292 ± 16	6
4 × 200[7]/12 hours	48	+	303 ± 16	324 ± 19	5

[1] Recalculated on the basis of a dose = 10^8 counts.
[2] Coprophagy prevented except where indicated.
[3] All animals fed Rockland diet.
[4] Indicates trace.
[5] Standard deviation of the mean.
[6] Indicates activity not determined.
[7] Coprophagy not prevented.

TABLE 5

Specific activity of thiamine (counts/μg/minute) in intestinal contents after injection of $S^{35}O_4^=$ into cecum of adult conventional rats[1,2]

Dose	Time of sacrifice (hours)	Diet	Cecum	Feces	No. of animals
μc					
50	3	L-356	360 ± 23[3]	—[4]	3
500	5	L-356	186 ± 23	—	3
200	7	Rockland	512 ± 81	—	4
200	24	Rockland	256 ± 42	165 ± 31	6

[1] Recalculated on the basis of a dose = 10^8 counts.
[2] Coprophagy prevented.
[3] Standard deviation of the mean.
[4] Indicates activity not determined.

a precursor, and again points to the cecum as the main site of synthesis. The specific activity observed in the colon and in the feces at best is equal to or otherwise slightly lower than the specific activity noted in the cecum. After administration of the tracer by stomach tube (table 4) the specific activity observed in the small intestine is much lower than that in the cecum. This radioactivity may be due to flora synthesis but could also be caused by reverse peristalsis from the cecum. Further analysis of table 4 shows that the final specific activity reached by the thiamine fraction is substantially higher if 4 doses of 200 μc are given at 12-hour intervals rather than the administration of a single 200 μc dose. A more continual supply therefore insures a higher degree of incorporation of the tracer into the vitamin B_1 molecule. Injection of the sulfate directly into the cecum permits the tracer to reach the main site of bacterial synthesis without loss, and results in a much higher specific activity than found after administration by stomach tube (tables 4 and 5). The data in table 5 also indicate that, after cecal injection, maximum activity is reached approximately 7 hours after administration of the sulfate.

The specific activity of the vitamin B_1 in the cecum was high after both cecal injection and prolonged administration by stomach tube. Since the heart is known to contain a relatively large amount of thiamine and since the liver is one of the first organs in which this vitamin should appear after absorption from the gut and transportation via the portal system, these organs were analyzed for radioactive thiamine. In all experiments, however, both hearts and livers were for all practical purposes devoid of any labeled thiamine. This suggests that the flora-synthesized thiamine was not available to the host to

any appreciable extent—not even in the 48 hour experiment in which coprophagy was allowed. Assuming that, especially after administration of $S^{35}O_4^=$ by stomach tube, most of the tracer is absorbed from the intestinal tract, the above observation also excludes tissue synthesis of a pattern found in intestinal bacteria.

One restriction has to be made in all our conclusions, namely, that the present experiments do not exclude the theoretical possibility of a thiamine synthesis using nonlabeled precursors and that this particular synthesis could lead to an absorbable form of thiamine.

Earlier workers had come to the conclusion that flora-synthesized thiamine was bound in the bacterial cell rather than being released into the surrounding medium (Abdel-Salaam and Leong, '38). Our data show that after centrifugation of a suspension of cecal contents in saline at 1600 rpm, the supernatant contains two to three times more thiamine (6.6 μg) than the residue (2.6 μg). This indicates that the vitamin B_1 (most of it apparently formed *in situ* as indicated in table 3) is not firmly bound to cells or cell fragments. The data in table 6 show that, at all times after cecal administration of the tracer, the ratio of the specific activity of thiamine in the supernatant to that in the cecal residue is approximately one, suggesting that the thiamine fractions in the supernatant and residue have the same history. If thiamine is formed originally in the bacterial cell, it is readily released into the environment.

Since the hearts and livers were devoid of detectable radioactive thiamine, there was reason for speculation as to the form in which the thiamine existed in the cecum. Therefore a determination was made of the amount of free, bound (detectable by the thiochrome method only after treatment of the sample with papain

TABLE 6

Specific activity (SA) of supernatant versus specific activity of residue of homogenized cecal contents (in saline) after administration of $S^{35}O_4^=$ into cecum of adult conventional rats[1]

Time after first injection	Dose	Diet	No. of animals	Av. SA of supernatant / SA of residue
	μc			
3	50	L-356	3	0.87
5	500	L-356	3	1.00
7	200	Rockland	4	1.19
24	200	Rockland	2	0.90
48	4×200	Rockland	4	0.95
				Av. = 0.98 ± 0.06[2]

[1] Centrifugation at 1600 rpm for 15 minutes.
[2] Standard deviation of the mean.

TABLE 7

Thiamine in supernatant fraction of conventional rats after centrifugation of homogenized cecal contents at 1600 rpm (450 × G) and subsequent centrifugation of this supernatant at 40,000 rpm (105,000 × G)[1,2]

Animal no.	Centrifugation rate for 15 minutes					
	At 1600 rpm			At 40,000 rpm		
	Total	Free	Bound[3]	Total	Free	Bound[3]
	μg	μg	μg	μg	μg	μg
1	4.4	0	4.4	3.4	0	3.4
2	3.1	0	3.1	2.6	0	2.6
3	1.8	0	1.8	1.5	0	1.5

[1] All operations conducted at 0°C.
[2] All animals fed Rockland diet.
[3] Bound thiamine was freed by treatment with papain and takadiastase prior to determination.

and takadiastase [see methods]) and total thiamine in the cecal supernatant after the contents were dispersed in the cold by shaking, followed by cold centrifugation at 1600 rpm (450 × G). Further analyses were made after subsequent centrifugation of this supernatant at 40,000 rpm (105,000 × G). The results in table 7 show the absence of any free thiamine in the supernatants. Furthermore, the bound thiamine in the supernatant decreased after centrifugation at 40,000 rpm. These data suggest that the flora-synthesized thiamine in the cecum is not in a free state and that at least part of it is bound to a moiety of high molecular weight, presumably a carboxylase, which is carried down at centrifugation rates of 40,000 rpm. Experiments now in progress, designed to study the properties of the flora-synthesized thiamine, have shown that, concomitant with the decrease in thiamine, carboxylase activity in the cecal supernatant is reduced upon centrifugation at 40,000 rpm. If the thiamine complex is a high molecular weight substance, this might well account for the lack of direct absorption of the vitamin from the digestive tract and subsequent utilization by the host animal. The experiments, where the oral administration of antibiotics to animals fed a thiamine deficient diet clearly indicated that more thiamine became available to the host (Jones and Baumann, '55; Scott and Griffith, '57; Wostmann et al., '58), might indicate that this flora-synthesized thiamine was available in a more absorbable form.

The above data and sensitivity of the methods described seem to exclude a contribution of flora-synthesized thiamine to the host of more than 0.5 μg per day when a complete diet is given and coprophagy is prevented. But even when coprophagy was permitted and the specific activity of the intestinal thiamine fraction increased over a 48-hour period to a value of approximately 300 counts/μg/min., no radiothiamine was observed in heart and liver thiamine. Apparently even under these circumstances the contribution of flora-synthesized thiamine to the host was low and relatively unimportant.

SUMMARY

$S^{35}O_4^=$ was administered by stomach tube and cecal injection to adult rats to study the availability of flora-synthesized thiamine. The flora of the digestive tract was found to incorporate radiosulfate into the *in situ* formed thiamine molecule, possibly via the cysteine or methionine routes.

The main site of microbial synthesis was in the cecum, although the possibility of synthesis in the small intestine could not be entirely excluded. Little or no absorption of the flora-synthesized radiothiamine occurred as shown by the lack of radiothiamine in hearts and livers. The contribution of the intestinal flora towards the thiamine requirement of the host thus seems at best low and relatively unimportant. Since most radiosulfate is absorbed as such from the intestinal tract, the negative findings in hearts and livers also exclude tissue synthesis of a pattern found in intestinal bacteria.

Upon centrifugation of a homogenate of cecal contents at 1600 rpm, the cecal supernatant contained two to three times more thiamine than the residue, although the specific activity of the thiamine of each was approximately the same. The thiamine in the cecum appeared to be tied to a large (possibly apo-coenzyme) complex. This, indeed, might account for lack of utilization of flora-synthesized thiamine by the host animal.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Dora Kan and Morris Wagner.

LITERATURE CITED

Abdel-Salaam, A., and P. C. Leong 1938 Synthesis of vitamin B_1 by intestinal bacteria of the rat. Biochem. J., *32:* 958.

Bandelin, F. J., and J. V. Tuschoff 1953 Colorimetric determination of thiamine in pharmaceutical products. Anal. Chem., *25:* 1198.

Barnes, R. H., E. Kwong, K. Delany and G. Fiala 1960 The mechanism of the thiamine-sparing effect of penicillin in rats. J. Nutrition, *71:* 149.

Bouman, J. 1948 Aneurine determination in potatoes. Internat. Vitaminforsch. Ztschr., *29:* 386.

Guerrant, N. B., and R. A. Dutcher 1934 Some effects of the composition of the diet on the vitamin B and the vitamin G requirement of the growing rat. J. Nutrition, *8:* 397.

Guerrant, N. B., R. A. Dutcher and L. F. Tomey 1935 The effect of the type of carbohydrate on the synthesis of the B vitamins in the digestive tract of the rat. J. Biol. Chem., *110:* 233.

Guerrant, N. B., R. A. Dutcher and R. A. Brown 1937 Further studies concerning the formation of the B-vitamins in the digestive tract of the rat. J. Nutrition, *13:* 305.

Jones, J. D., and C. A. Baumann 1955 Relative effectiveness of antibiotics in rats given limited B vitamins by mouth or by injection. Ibid., 57: 61.

Kulwich, R., L. Struglia and P. B. Pearson 1957 The metabolic fate of S^{35} in sheep. Ibid., *61:* 113.

Larner, J., and R. E. Gillespie 1957 Gastrointestinal digestion of starch. III. Intestinal carbohydrase activities in germ-free and nongerm-free animals. J. Biol. Chem., 225: 279.

Mameesh, M. S., R. E. Webb, H. W. Norton and B. C. Johnson 1959 The role of coprophagy in the availability of vitamins synthesized in the intestinal tract with antibiotic feeding. J. Nutrition, 69: 81.

Mitchell, H. K., and E. R. Isbell 1942 Intestinal bacterial synthesis as a source of B vitamins for the rat. Univ. Texas Publ. no. 4237, p. 125.

Pelc, S. R., and A. Glucksmann 1955 Sulphate metabolism in the cartilage of the trachea, pinna and xiphoid process of the adult mouse as indicated by radioautographs. Exp. Cell Res., *8:* 336.

Ping-Pan, H. 1956 A simple apparatus for rapid study of paper partition chromatography. Arch. Biochem., 64: 311.

Reyniers, J. A. 1956 Proceedings of Third International Congress of Biochemistry, Brussels, 1955 (ed., C. Liebecq). Academic Press Inc., New York, p. 458.

Scott, E. M., and J. V. Griffith 1957 A comparative study of thiamine-sparing agents in the rat. J. Nutrition, *61:* 421.

Shimomura, F., J. Ogata, H. Nakayama and R. Hayashi 1957 Thiamine biosynthesis in bacteria. J. Vitaminol., *3:* 96.

Siliprandi, D., and N. Siliprandi 1954 Separation and quantitative determination of thiamine and thiamine phosphoric esters and their preparation in pure form. Biochim. biophys. acta, *14:* 52.

Wostmann, B. S., P. L. Knight and J. A. Reyniers 1958 The influence of orally-administered penicillin upon growth and liver thiamine of growing germfree and normal stock rats fed a thiamine-deficient diet. J. Nutrition, 66: 577.

Yamada, K., S. Sawaki and S. Kayami 1957 The incorporation of DL-methionine-S^{35} into thiamine. J. Vitaminol., *3:* 73.

Tocopherol Content of Maternal and Fetal Rat Tissues as Related to Vitamin E Intake during Gestation[1]

DOROTHY WEI CHENG, KARL G. BRAUN, BERNARD J. BRAUN AND K. H. UDANI

Department of Anatomy, State University of Iowa College of Medicine, Iowa City, Iowa

Previous studies have shown that congenital malformations may be produced in vitamin E-deficient rats fed a semisynthetic ration under certain specific conditions (Thomas and Cheng, '52; Cheng and Thomas, '53).[2] Since rats receiving a pure synthetic vitamin E-deficient ration can also produce young under identical conditions (Cheng et al., '60), it seemed worthwhile to determine chemically the level of vitamin E in some maternal and fetal tissues in the hope of gaining some insight into the manner in which the rat utilized and stored the vitamin under vitamin E-sufficient, vitamin E-deficient and marginally E-deficient conditions. Giroud and Boisselot ('60) showed that in pantothenic acid deficiency teratogeny was due to a slight degree of maternal deficiency. In the present study any possible differences between the tissue tocopherol levels of the normal and abnormal fetuses were investigated.

Tocopherol serum levels of various animals are summarized in table 1, while the values for liver and muscle are given in table 2. The tocopherol level in the tissues varies in different animals and under different dietary conditions. Generally, graded doses of the vitamin cause a proportional increase in the tissue vitamin levels, but the liver takes the main share of the ingested vitamin (Bratzler et al., '50). Quaife ('52) reported that about 10% of the dose was in the liver of the rats, but the stores did not seem to be maintained for long. In the human, vitamin E occurs mainly in the adipose tissue which contains 65 to 75% of the body's vitamin store, and only a small portion in the liver (Kodicek, '54). Premature infants have higher tocopherol reserves than the mature new-born. The amount of tocopherol increases with age, mostly in the body fat. In advanced age, however, a sudden drop occurs in the vitamin E content in the liver, body fat and elsewhere.[3]

MATERIAL AND METHODS

The Holtzman rats used in these experiments were fed the pure synthetic vitamin E-deficient ration (PSR1, Cheng et al., '60) from weaning until the end of gestation. The breeding procedure has been reported before (Cheng and Thomas, '53; Cheng et al., '60). As soon as the rats were mated they were separated into different groups. Rats in the positive control group that had received 2 mg of *dl*-α-tocopheryl acetate (in 1 ml of corn oil by stomach tube) daily during the first 5 days of gestation were considered vitamin E-sufficient. Those in the negative control group without any supplementation were vitamin E-deficient, and those in the experimental groups with either 2 or 4 mg of vitamin E supplementation on the 10th day of gestation were marginally deficient in vitamin E. At term the blood of both the mother rats and the fetuses was obtained by heart puncture. For the fetuses the blood usually had to be pooled from two or three animals for a large enough sample for the tocopherol

Received for publication December 31, 1960.

[1] Supported by grant no. RG-4993, U. S. Public Health Service.

[2] Cheng, D. W. 1954 A study of the occurrence of teratogeny in vitamin E-deficient rats and associated abnormalities in blood and tissues. Ph.D. Thesis. Iowa State College, Ames.

[3] Mason, K. E., M. Y. Dju and L. J. Filer, Jr. 1952 Distribution of tocopherols in human tissues. Federation Proc., *11:* 449 (abstract).

TABLE 1

Vitamin E content of the sera of various animals

Animal species	Ration or ration plus prepartal vitamin supplement	Duration, days	Vitamin E				References
			Mother or adult	Newborn	Umbilical vein	Placenta	
			mg/100 ml				
Man			1.70	0.340			Straumfjord and Quaife, ('46)
			1.82	0.380			Wright et al. ('51)
				0.096			Abderhalden ('45)
			0.58	0.353	0.283		Gerloczy et al. ('51)
			0.20–1.70				Faaborg-Andersen ('46)
			1.07				Quaife et al. ('49a)
			1.51				Scrimshaw et al. ('49)
			1.0–1.2				Dju et al. ('58)
	Normal				0.220		Neuweiler ('49)
	Normal + 90 mg vitamin E	before delivery			0.656		
						0.77	Athanassiu ('46)
						0.61	Dju et al. ('52)
Cattle	Barn		0.276	0.044			Parrish et al. ('50)
	Barn + 4 gm mixed vitamin E daily	0–28 prepartum	1.003	0.068			
	Barn + 10 gm mixed vitamin E daily	0–28 prepartum	1.101	0.079			
	Vitamin E-deficient		0.25				Kachmar et al. ('50)
	Vitamin E-deficient + 5 mg Vitamin E/kg body weight/day		0.69				
	Winter		0.10–0.20				Van der Kaay et al. ('49)
	Summer		0.80–1.00				Blaxter et al. ('52)

Pig	Vitamin E-deficient	75	0		Bratzler et al. ('50)
	Vitamin E-deficient + 28.7 mg mixed vitamin E/kg body weight	75	0.25		
	Vitamin E-deficient + 55.1 mg mixed vitamin E/kg body weight	75	0.67		
	Vitamin E-deficient + 110.2 mg mixed vitamin E/kg body weight	75	0.59		
	Vitamin E sufficient	growth through pregnancy		0.120	Whiting and Loosli ('48)
	Vitamin E-sufficient + 80 mg mixed vitamin E/100 pounds body weight daily	growth through pregnancy		0.101	
Chick	Vitamin E-deficient		0.10–0.40		Zacharias et al.[1]
	Vitamin E-deficient + 21 mg E/week		1.50		
Ewe			0.21–0.30		Meunier et al. ('48)
	Vitamin E-deficient	growth through pregnancy		0.020	Whiting and Loosli ('48)
	Vitamin E-deficient + 80 mg mixed vitamin E/100 pounds body weight daily	growth through pregnancy		0.094	
Goat	Vitamin E-deficient	growth through pregnancy		0.016	Whiting and Loosli ('48)
	Vitamin E-deficient + 80 mg mixed vitamin E/100 pounds body weight daily	growth through pregnancy		0.065	
Horse			0.10–0.20		Van der Kaay et al. ('49)
Rat, male			0.70		Quaife et al. ('49b)

[1] Zacharias, L., P. Goldhaber and V. E. Kinsey 1950 Effects of dietary supplements on plasma tocopherol levels and vitamin E deficiency symptoms in chicks. Federation Proc., 9: 375 (abstract).

TABLE 2

Vitamin E content of the liver and skeletal muscle of various animals

Animal species	Ration or ration plus prepartal vitamin supplement	Duration, days	Vitamin E		References
			Liver	Muscle	
			mg/100 gm		
Man			0.46		Abderhalden ('45)
			1.10	1.20	Dju et al. ('58)
				2.42	Kaunitz and Beaver ('46)
Calf			0.60		Dju et al. ('58)
			1.38		Whiting and Loosli ('48)
Ox			1.0–1.4		Quaife and Dju ('49)
			0.95	0.59	Karrer et al. ('40)
Beef			1.78	0.26	Swick and Baumann ('52)
			1.40		Harris et al. ('50)
				0.25	Kofler ('45)
Pig	Vitamin E-deficient	75	0.29	0.37	Bratzler et al. ('50)
	Vitamin E-deficient + 28.7 mg mixed vitamin E/kg body weight	75	1.20	1.82	Bratzler et al. ('50)
	Vitamin E-deficient + 55.1 mg mixed vitamin E/kg body weight	75	42.35	4.16	Bratzler et al. ('50)
	Vitamin E-deficient + 110.2 mg mixed vitamin E/kg body weight	75	20.81	12.49	Bratzler et al. ('50)
	Vitamin E-sufficient	growth through pregnancy	2.47		Whiting and Loosli ('48)
	Vitamin E-deficient + 80 mg mixed vitamin E daily/100 pounds body weight	growth through pregnancy	2.64		Whiting and Loosli ('48)
Chick			0.30		Kodicek ('54)
Kids	Vitamin E-deficient	growth through pregnancy	1.04		Whiting and Loosli ('48)
	Vitamin E-deficient + 80 mg mixed vitamin E daily/100 pounds body weight	growth through pregnancy	1.31		Whiting and Loosli ('48)
Lambs	Vitamin E-deficient	growth through pregnancy	2.83		Whiting and Loosli ('48)
	Vitamin E-deficient + 80 mg mixed vitamin E daily/100 pounds body weight	growth through pregnancy	3.00		Whiting and Loosli ('48)
Horse			1.32	0.53	Karrer et al. ('40)
Rabbit			0.30		Kofler ('45)
	Vitamin E-deficient		0.94	0.57	Hines and Mattill ('43)
	Vitamin E-deficient + 100 mg vitamin E daily		8.68	2.81	Hines and Mattill ('43)
Rat	Vitamin E-deficient		0.37		Swick and Baumann ('52)
Male	Vitamin E-deficient + 1 mg vitamin E daily		2.94	0.40	Swick and Baumann ('52)
Male	Vitamin E-deficient + 1 mg vitamin E daily		2.52	1.33	Quaife et al. ('49b)
				2.09	Kaunitz and Beaver ('46)
Male	Vitamin E-deficient		2.26	0.48	Hines and Mattill ('43)
Male	Vitamin E-deficient + 100 mg vitamin E daily		4.23	1.19	Hines and Mattill ('43)

determinations. The blood samples in the 6 × 50-mm tubes were allowed to clot and then centrifuged to obtain the sera which were frozen at −20°C until the time of assay. The liver and the thigh muscles of the mother rats together with the liver and the carcass of the young were weighed, placed in 95% ethanol and stored in the refrigerator.

For the liver, muscle and carcass vitamin E determinations the method of Swick and Baumann ('52) was used. For the total tocopherols in serum the micromethod of Quaife et al. ('49a) was adopted with minor modifications in the quantities of reagents used, as follows:

Procedure for standard curve. One-hundred-and-eighty mm³ of standard solution of α-tocopherol in absolute ethanol were put in 6 × 50-mm tubes fitted with corks covered with aluminum foil; 60 mm³ of distilled water were added to this solution; 240 mm³ of xylene were then added and the tube was shaken until the mixture appeared homogeneous. The tubes were centrifuged for 10 minutes at 3000 rpm; 160 mm³ of supernatant xylene were then transferred to another 6 × 50-mm tube and to it were added 160 mm³ of 2,2′-bipyridine (0.120 gm/100 ml). The contents were thoroughly mixed; 240 mm³ of this solution were transferred to Beckman cells and the absorbance read at 460 mμ to determine the amount of carotenoids in solution. Then 50 mm³ of ferric chloride solution (120 gm/100 ml absolute ethanol) were added and the absorbance read at 520 mμ after an interval of three minutes for the development of the Emmerie-Engel reaction color (Emmerie and Engel, '43). A linear graph of absorbance, at 520 mμ, against quantity of α-tocopherol was then plotted after a series of readings was made on solutions ranging in concentration from 0.125 to 2 mg per ml.

For serum tocopherol determinations the same procedure was followed except that distilled water was replaced by the serum sample and the standard solution had a concentration of 0.5 mg of α-tocopherol per ml. The added tocopherol was necessitated by the low level of this vitamin in the serum. In the final calculations due corrections were made for the added amount of tocopherol and the carotenoids in the blood.

From 5 additional experiments to determine the precision of this analytical procedure, 0.125 to 2 mg/ml of α-tocopherol were added to small amounts of sample and an average of 96.5 ± 1.7% of the added tocopherol was recovered.

RESULTS AND DISCUSSION

The results of the chemical assays are summarized in table 3.

Maternal serum tocopherol content. The serum tocopherol content was lowest in the rats of the positive control group, highest in the negative control group, and intermediate in the 2- and 4-mg experimental groups. The difference between the positive and negative groups was significant at the 5% level, whereas that between the positive and 4-mg group was significant at the 1% level. The values of 0.637 to 0.761 mg per 100 ml were similar to the 0.70 mg per 100 ml reported by Quaife et al. ('49b; table 1) for the male rat. As shown in table 1, the serum tocopherol level reflects the dietary intake of the individual. Therefore, it is not unexpected to find significant differences between some of the groups in these experiments (table 3). Speculation on the reasons for the higher serum tocopherol level in the vitamin E-deficient mother rats than in the vitamin E-sufficient ones suggests the following. The higher level here might reflect nonutilization or reduced demand of the vitamin since there were no live young at term in this group. Also, the rats in the positive group with an average of 8 or 9 live young would have the lowest serum tocopherol level while those in the experimental groups with only two or three live young at term would show intermediate levels of serum tocopherol, as observed.

Maternal liver tocopherol content. The liver tocopherol level of rats in the positive control group was the lowest, whereas those of animals fed either a vitamin E-deficient diet or on the experimental regimens were higher. As in the maternal serum tocopherol level, this phenomenon could be explained by the fact that the rats in the positive group gave birth to the average number of normal young. The

TABLE 3

Vitamin E content of maternal and fetal rat tissues under vitamin E-sufficient, vitamin E-deficient and marginally vitamin E-deficient conditions

Group	Dose of *dl*-α-tocopheryl acetate/day	Given on day(s) of gestation	Maternal vitamin E			Fetal vitamin E					
			Serum	Liver	Skeletal muscle	Serum		Liver		Carcass	
						Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
	mg		*mg/100 ml*	*mg/100 gm*		*mg/100 ml*		*mg/100 gm*		*mg/100 gm*	
Positive control	2	1–5	0.637 ± 0.063[1] (2)[2]	4.74 ± 2.43 (12)	0.529 ± 0.206 (12)	0.728 ± 0.115[7] (8)		0.825 ± 0.312 (6)		0.186 ± 0.079 (8)	
Experimental 1	2	10	0.720 ± 0.088 (12)	6.71 ± 2.99[6] (14)	0.463 ± 0.272 (13)	0.827[7] (1)	0.629 ± 0.029[7] (2)	2.53 ± 1.90 (7)	0.941 ± 0.941 (3)	0.23 ± 0.40 (7)	0.13 ± 0.07 (5)
Experimental 2	4	10	0.725 ± 0.149[3] (31)	7.65 ± 2.29[3] (13)	0.347 ± 0.156[4] (13)	0.226[7] (1)	0.800 ± 0.141[7] (4)	0.989 ± 0.709 (4)	0.525 ± 0.353 (2)	0.300 ± 0.149 (3)	
Negative control	0	—	0.761 ± 0.119[5] (20)	6.03 ± 4.07 (13)	0.355 ± 0.122[5] (12)						

[1] Standard deviation.

[2] Figures in parentheses represent the number of samples.

[3] $P < 0.01$ when compared with corresponding positive control group.

[4] $P < 0.05$ when compared with corresponding positive control group.

[5] $P < 0.05$ when compared with corresponding positive control group.

[6] $P < 0.01$ when compared with corresponding positive control group.

[7] Pooled samples.

liver tocopherol content of rats with 2 mg of vitamin E supplementation on the 10th day was significantly (1% level) higher than that of rats in the negative group. The liver tocopherol value was highest in the group with 4 mg of E supplementation. The difference between this and that of the positive group was significant at the 1% level.

The uptake of ingested tocopherol by liver and blood as a function of dosage by the rat (Bolliger and Bolliger-Quaife, '56), calves, lambs and pigs (Rousseau et al., '57), and the chick (Bunnell, '57) is also reflected here in the negative control, experimental groups receiving 2 and 4 mg. The linear relationship between liver tocopherol content and tocopherol dosage is evident (table 3). The liver is a good storage depot for vitamin E (Mason, '44), surpassed only by the adipose tissue and especially the endocrine organs (Quaife et al., '49b). Apparently the tocopherol in the liver is very labile since it can show variations according to intake and gestational needs as shown in our experiments.

Tocopherol content of maternal skeletal muscle. The tocopherol content of the maternal skeletal muscle, in general, was much lower than that of the liver, about 1/9th as much in the positive control group to 1/20th in the 4-mg experimental group. This ratio is much smaller than that observed in normal human skeletal muscle tissue, or in the male rat where the vitamin E level of the muscle was approximately half that of the liver (Quaife et al., '49b). The tocopherol value of the muscle of the rat in the positive control group was the highest, and that of the negative group was the lowest. The difference between these was significant at the 5% level. The value observed for the positive control group compares favorably with that reported by Swick and Baumann ('52) for the muscle of the male rat (table 2). The muscle tocopherol content of rats with 4-mg supplementation was about the same as that of the negative group. The difference between the values of the 4-mg and the positive groups was also significant at the 5% level. The muscle tocopherol level of rats receiving 2-mg supplementation was intermediate between the values for the negative and positive groups.

From the foregoing, it appears that the vitamin E level of the maternal skeletal muscle very nearly reflects the vitamin intake of the animal. Compared with that in the liver, however, it probably is in a very stable state and contributes little, if any, to the fulfillment of the vitamin E needs of gestation.

When examined as a whole, the data on maternal serum, liver and muscle show that the tocopherol levels of the serum and liver indicate the same trend, whereas those of the muscle manifest the opposite trend. The results seem to indicate that the tocopherol needed for fetal development came from the liver instead of the muscle since the liver tocopherol of rats in the positive group which gave birth to the normal average number of live young, was much reduced, while the muscle tocopherol remained high.

The tocopherol level of the fetal serum. From the scanty literature (table 1) it seems that in humans as well as in cattle the tocopherol content of the maternal serum is 5 or 6 times that of the fetal serum (Straumfjord and Quaife, '46; Parrish et al., '50; Wright et al., '51). In our experiments, however, except for one incidence, that of the normal serum of the 4-mg group, the tocopherol levels of the fetal sera were approximately the same as those of the maternal sera (table 3). The tocopherol level of the fetal serum of the positive control group was slightly higher than that of the corresponding maternal serum. In the experimental groups the averages for the total fetal serum tocopherol levels were slightly lower than those of the corresponding maternal sera. Excepting the 4-mg group, none of the differences between the maternal and fetal sera was statistically significant. Thus our results do not corroborate the view that in the rat the placental transfer of vitamin E is exceedingly limited (Mason and Bryan, '40).

In the 2-mg group the serum tocopherol level of the normal fetuses was slightly higher than than of the abnormal ones, while the reverse was true for the 4-mg group. The differences between the corresponding groups were not significant

statistically, however. Thus no trend could be discerned in this respect.

Tocopherol level of the fetal liver. In the positive group the tocopherol level of the fetal liver was slightly less than 1/6th of the maternal liver level. In the 2-mg group the total average value reached a level of slightly less than 1/4th of the maternal level. In the 4-mg group it was only approximately 1/15th that of the maternal value. Compared with the normal fetuses, the average tocopherol level of the abnormal ones was slightly, though not significantly, lower in the 2-mg group, but about the same in the 4-mg group. In human monsters at term, Dju et al. ('52) reported that the liver tocopherol level was 0.64 mg per 100 gm. This figure is quite close to the 0.525 mg per 100 gm in the liver of the abnormal young from the 4-mg group of our experiments.

Tocopherol level of the fetal carcass. Since the bulk of the fetal skeletal muscle was so small, it was deemed advisable to determine the tocopherol level of the carcass instead. The values obtained were very small. The average value for the 2-mg group was approximately the same as that for positive control group. When compared with the normal carcasses, the abnormal ones contained much less tocopherol. The difference, however, was not statistically significant.

From the data presented it seems that, compared with the normal fetuses, the abnormal ones had slightly lower tocopherol level in serum, liver and carcass. Furthermore, our results seem to confirm Giroud and Boisselot's ('60) observations in maternal pantothenic acid deficiency that only a slight degree of maternal vitamin deficiency would produce congenital abnormalities in the young.

SUMMARY

Female Holtzman rats were reared from weaning until the end of gestation on a pure vitamin E-deficient ration. In the positive control group each rat was given daily 2 mg of *dl*-α-tocopheryl acetate during the first 5 days of gestation. In the experimental groups, either 2 or 4 mg of the vitamin was given on the 10th day of gestation, whereas in the negative control group no vitamin E supplement was supplied. All rats were autopsied on the 21st day of gestation. Chemical assays of the tocopherol content of maternal and fetal serum and liver as well as the maternal skeletal muscle and the fetal carcasses were carried out. A total of 265 determinations was performed in order to study the changes in the tocopherol levels during pregnancy and the production of abnormal young.

The maternal serum tocopherol level was lowest in the vitamin E-sufficient group, highest in the vitamin E-deficient group and intermediate in the groups with abnormal young. The maternal liver tocopherol generally followed the same trend as the serum with slight modifications, whereas the tocopherol level of the maternal skeletal muscle showed the opposite trend, namely, it was highest in the vitamin E-sufficient group, lowest in the vitamin E-deficient group and intermediate in the groups with abnormal young. There were no significant differences between the tocopherol levels of fetal sera, liver, and carcasses from different groups. The tocopherol levels of the abnormal fetal sera, liver and carcasses were slightly, though not significantly, lower than those of the normal ones from the corresponding groups.

ACKNOWLEDGMENTS

We are indebted to Dr. N. S. Halmi for criticism of the manuscript and the late Dr. Edmund W. Cheng for their suggestions during the course of the experiments.

LITERATURE CITED

Abderhalden, R. 1945 Der Tokopherolgehalt in den Organen von Menschlichen Föten und Neugeborenen. Ztschr. Vitaminforsch., *16:* 319.

Athanassiu, G. 1946 Über den Gehalt der Placenta an Vitamin E. Klin. Wochnschr., *24:* 170.

Blaxter, K. L., F. Brown and A. M. MacDonald 1952 Muscular abnormalities of newborn calves in relation to the tocopherol nutrition of their dams. Brit. J. Nutrition, *6:* 1.

Bolliger, H. R., and M. L. Bolliger-Quaife 1955 Analytical methods and their use in evaluation of vitamin E. Third International Congress on Vitamin E., p. 30.

Bratzler, J. W., J. K. Loosli, V. N. Krukovsky and L. A. Maynard 1950 Effect of the dietary level of tocopherols on their metabolism in swine. J. Nutrition, *42:* 59.

Bunnell, R. H. 1957 The vitamin E potency of alfalfa as measured by the tocopherol content of the liver of the chick. Poultry Sci., *36:* 413.

Cheng, D. W., and B. H. Thomas 1953 Relationship of time of therapy to teratogeny in maternal avitaminosis E. Proc. Iowa Acad. Sci., 60: 290.

Cheng, D. W., T. A. Bairnson, A. N. Rao and S. Subbammal 1960 Effect of variations of rations on the incidence of teratogeny in vitamin E-deficient rats. J. Nutrition, 71: 54.

Dju, M. Y., K. E. Mason and L. J. Filer 1952 Vitamin E in human fetuses and placentae, Étude Néo-Natales, 1: 49.

——— 1958 Vitamin E. (Tocopherol in human tissues from birth to old age.) Am. J. Clin. Nutrition, 6: 50.

Emmerie, A., and C. Engel 1943 Tocopherol (vitamin E) content of foods and its chemical determinations. Ztsch. Vitaminforsch., 13: 259.

Faaborg-Andersen, K. 1946 The tocopherol content of the blood. Nord. Med., 32(42): 2401; cited in Chem. Abstr., 4: 3189 f.

Gerloczy, F., Bencze, J. Szenasy and D. Kuncz 1951 Examination of the vitamin-E barrier of the placenta. Experientia, 7: 427.

Giroud, A., and J. Boisselot 1960 Specificity and slightness of the teratogenic pantothenic acid deficiency. Anat. Rec., 136: 198.

Harris, P. L., M. L. Quaife and W. J. Swanson 1950 Vitamin E content of foods. J. Nutrition, 40: 367.

Hines, L. R. and H. A. Mattill 1943 The chemical determination of tocopherols in liver and muscle; tocopherol in urine and feces. J. Biol. Chem., 149: 549.

Kachmar, J. F., P. D. Boyer, T. W. Gullickson, E. Liebe and R. M. Porter 1950 Vitamin E in the nutrition of cattle. II. Vitamin E levels in the blood, vitamin A and carotene utilization and other chemical studies. J. Nutrition, 42: 391.

Karrer, P., W. Jaeger and H. Keller 1940 Tocopherol-Bestimmungen in tierischen Organen. Helvet. chim. acta, 23: 464.

Kaunitz, H., and J. J. Beaver 1946 Tocopherol content of skeletal muscle: comparison of chemical and bioassay methods. J. Biol. Chem., 166: 205.

Kodicek, E. 1954 Storage of vitamins in liver. Proc. Nutrition Soc., 13: 125.

Kofler, M. 1945 Die fluorometrische Methode zur Bestimmung von Tocopherol. 3. Mitteilung. Die Bestimmung in Pflanzen und in tierischen Organen. Helvet. chim. acta, 28: 26.

Mason, K. E. 1944 Physiological action of vitamin E and its homologues. Vitamins and Hormones, 2: 116.

Mason, K. E., and W. L. Bryan 1940 Placental and mammary transfer of vitamin E in rat. J. Nutrition, 20: 501.

Meunier, P., R. Ferrando and P. Chenavier 1948 Le taux de tocopherol (vitamine E) sanguine du Mouton. Action de L'Huile de foie de Morue. C. r. soc. biol., 142: 525.

Neuweiler, W. 1949 Vitamin E-Übertritt von Mutter auf Kind. Internat. Ztschr. Vitaminforsch., 21: 83.

Parrish, D. B., G. H. Wise, C. E. Latschar and J. S. Hughes 1950 Effect of the prepartal diet of the cow on the placental and mammary transfer of tocopherols to the calf. J. Nutrition, 40: 193.

Quaife, M. L. 1952 The relation of tocopherol content of animal tissues to types and amounts of vitamin E fed. Congr. int. Biochim. II, Paris. Résumés des Communications, p. 221.

Quaife, M. L., and M. Y. Dju 1949 Chemical estimation of vitamin E in tissues and the tocopherol content of some normal human tissues. J. Biol. Chem., 180: 263.

Quaife, M. L., N. S. Scrimshaw and O. H. Lowry 1949a Micromethod for assay of total tocopherols in blood serum. Ibid., 180: 1229.

Quaife, M. L., W. J. Swanson, M. Y. Dju and P. L. Harris 1949b Vitamin E in foods and tissues. Ann. N. Y. Acad. Sci., 52: 300.

Rousseau, J. E., Jr., M. W. Dicks, R. Teichman, C. F. Helmbodt, E. L. Bacon, R. M. Prouty, K. L. Dolge, H. D. Eaton, E. L. Jungherr and G. Beall 1957 Relationships between plasma, liver and dietary tocopherol in calves, lambs and pigs. J. Animal Sci., 16: 612.

Scrimshaw, N. S., R. B. Greer and R. L. Goodland 1949 Serum vitamin E levels in complications of pregnancy. Ann. N. Y. Acad. Sci., 52: 312.

Straumfjord, J. V. and M. L. Quaife 1946 Vitamin E levels in maternal and fetal blood plasma. Proc. Soc. Exp. Biol. Med., 61: 369.

Swick, R. W., and C. A. Baumann 1952 Chemical assay for tocopherol in animal materials. Anal. Chem., 24: 758.

Thomas, B. H., and D. W. Cheng 1952 Congenital abnormalities associated with vitamin E malnutrition. Proc. Iowa Acad. Sci., 59: 218.

Van der Kaay, F. C., G. H. B. Teunissen, A. Emmerie and M. van Eekelen 1949 The tocopherol serum level of cows and horses in relation to reproduction. Ann. N. Y. Acad. Sci., 52: 276.

Whiting, F., and J. K. Loosli 1948 The placental and mammary transfer of tocopherols (vitamin E) in sheep, goats and swine. J. Nutrition, 36: 721.

Wright, S. W., L. J. Filer and K. E. Mason 1951 Vitamin E blood levels in premature and full term infants. Pediatrics, 7: 386.

Effect of Supplementary Lysine and Methionine on Enteritis Mortality, Growth and Feed Efficiency in Young Rabbits

R. B. CASADY,[1] R. A. DAMON[2] AND A. E. SUITOR[1]
U. S. Department of Agriculture, Fontana, California and Beltsville, Maryland

The importance of quality of protein in the diet of the rabbit has not been established, although some investigators feel that protein quality may be of minor importance because of the simple feeds that have been successfully utilized in rabbit rations (National Research Council, '54).

At the U. S. Rabbit Experiment Station it was decided recently to investigate the effects of supplementary amino acids on the incidence of enteritis mortality, and on growth and feed efficiency in young rabbits. For many years enteritis has been a principle cause of mortality among young domestic rabbits (Lund, '51), and all efforts to isolate the cause of this condition have been unsuccessful (Hagen, '56). Since the gross appearance of rabbits with enteritis suggests a toxic condition, and amino acids are known to be involved in detoxification, it was felt that amino acid supplementation might be effective in reducing the incidence of this condition.

Lysine and methionine were chosen for initial studies primarily because of their beneficial effects on growth and protein utilization in other animals (Acker et al., '59; Almquist, '57; Baron, '58; Germann et al., '58; Hays et al., '59; Squibb et al., '59). Although there may be reason to believe that intestinal synthesis of amino acids occurs in the rabbit (Casady and Gildow, '59), little is known about this problem, nor have any amino acid requirements been established for the rabbit (National Research Council, '54).

MATERIALS AND METHODS

This investigation involved two experiments.

General. Both experiments involved the feeding of mature females (does) and their young from mating of the does until the young were weaned at 56 days of age. The does were accustomed to their experimental rations before being mated, and were hand-fed restricted amounts until palpated for pregnancy 12 days following breeding. Those that failed to conceive continued to be fed the restricted amounts to maintain the desired physical condition until determined to be pregnant. Following positive palpation for pregnancy, the does were full-fed from hoppers for the remainder of the gestation period. Does and litters were full-fed throughout the suckling period.

Feed was weighed and recorded from conception of the does to weaning of the litter. Does were rebred 52 to 53 days following parturition. Insofar as possible, litters were equalized at 8 young each within three days following parturition. Young were weighed at 56 days of age. All animals that died during the test were autopsied to determine cause of death.

The does and their litters were housed in all-wire hutches equipped with wire-mesh floors and automatic watering systems.

Experiment 1. This test involved 9 treatments, 8 does and their litters, per treatment. The ration consisted of the pelleted stock ration used at the U. S. Rab-

Received for publication December 3, 1960.

[1] Sheep and Fur Animal Research Branch, Animal Husbandry Research Division, U. S. Rabbit Experiment Station, Fontana, California.

[2] Biometrical Services, Agricultural Research Center, Beltsville, Maryland.

bit Experiment Station, supplemented with lysine[3] and methionine[4] (table 1). The does on test reared two successive litters while fed the experimental rations.

Experiment 2. Two series of 4 treatments each were used, including 8 does and their litters, per treatment. The ration consisted of the pelleted stock ration used at the U. S. Rabbit Experiment Station supplemented with methionine only (table 1). The does on test reared three successive litters while fed the experimental rations.

TABLE 1

Supplementation of lysine (Ly) and methionine (M) in the basic stock ration[1]

Treatment	Lysine	Methionine
	%	%
Experiment 1		
1 Ly_1 M_1 (control)	0.0	0.0
2 Ly_1 M_2	0.0	0.1
3 Ly_1 M_3	0.0	0.2
4 Ly_2 M_1	0.2	0.0
5 Ly_2 M_2	0.2	0.1
6 Ly_2 M_3	0.2	0.2
7 Ly_3 M_1	0.4	0.0
8 Ly_3 M_2	0.4	0.1
9 Ly_3 M_3	0.4	0.2
Experiment 2		
1 M_1 (control)	—	0.0
2 M_2	—	0.1
3 M_3	—	0.2
4 M_4	—	0.4

[1] Percentage composition: suncured alfalfa hay meal, 40; soybean oil meal (44% protein), 18; ground barley, 18.5; linseed oil meal (expeller), 4; oats, 4; wheatmixed feed, 15; salt (NaCl), 0.5.

Analysis of data. The incidence of enteritis mortality, as determined by postmortem examination, was analyzed by chi-square tests in an approximate analysis of variance for both experiments. Where significant differences among levels of amino acids or treatments existed, additional orthogonal contrasts were made. If no significant differences existed, no further breakdown was made.

Analysis of weaning weights was made by the method of fitting constants in a least squares analysis. When significant differences existed between treatments, further breakdown of the data was made in order to find the source of treatment difference. Differences between levels of lysine and methionine were broken down

[3] L-Lysine monohydrochloride supplied by Chas. Pfizer and Company, Inc., Brooklyn, New York.

[4] DL-Methionine-feed grade supplied by U. S. Industrial Chemicals Company, New York.

TABLE 2

Incidence of enteritis mortality

	Experiment 1		Experiment 2	
	No. animals	Enteritis mortality	No. animals	Enteritis mortality
		%		%
Treatment 1 (control)	125	15.2	344	13.4
2	126	7.1	331	23.0
3	111	13.5	323	16.1
4	118	14.4	334	18.9
5	116	7.7		
6	94	14.9		
7	126	23.8		
8	97	11.3		
9	126	12.7		
Levels of lysine				
Ly_1	362	11.9		
Ly_2	328	12.2		
Ly_3	349	16.3		
Levels of methionine				
M_1	369	17.9		
M_2	339	8.5		
M_3	331	13.6		
Litters 1	519	9.1	490	15.9
2	520	17.9	469	14.9
3	—	—	373	23.9

into linear and quadratic effects. Significant differences among treatment means were separated using a multiple range test described by Duncan ('55).

Feed conversion data (pounds of feed per pound of gain) were analyzed by the same methods described for the weaning weights. In experiment 1 the sums of squares among levels of lysine were further broken down into linear and quadratic effects.

RESULTS AND DISCUSSION

In table 2 are shown the numerical values for the number of animals and the percentage of enteritis mortality by treatment, levels of lysine, and levels of methionine for both experiments. Chi-square tests of significance (table 3) showed highly significant differences among treatments in experiment 1. A partitioning of the variance among treatments indicated that differences among levels of methionine was the major source of variation. Further orthogonal contrasts showed that the curvilinear response to methionine treatment was highly significant. There were no significant differences between levels of lysine so no further breakdown was made. Also there was no apparent interaction between lysine and methionine. Highly significant differences, however, were observed between successive litters, wherein mortality was highest in second litters from individual does.

TABLE 3

Chi-square tests of significance of incidence of enteritis

Source	d.f.	χ^2
Experiment 1		
Treatments	8	20.141[1]
Levels of lysine (Ly)	2	3.696
Levels of methionine (M)	2	13.202[1]
Linear	1	2.756[1]
Quadratic	1	10.634[1]
Interaction (Ly × M)	4	3.242
Litters	1	17.366[1]
Experiment 2		
Treatments	3	11.534[1]
M_1 vs. M_2, M_3 and M_4	1	6.145[2]
M_2 vs. M_3 and M_4	1	4.519
M_3 vs. M_4	1	0.857
Litters	2	13.202[1]

[1] Significant at the 1% level of probability.
[2] Significant at the 5% level of probability.

TABLE 4

Least squares means of weaning weights

	Experiment 1	Experiment 2
	pounds	*pounds*
Treatment		
1 (control)	3.49	3.75
2	3.85	3.78
3	3.69	3.87
4	3.82	3.70
5	3.90	—
6	3.82	—
7	3.75	—
8	3.74	—
9	3.76	—
Litters		
1	3.67	3.67
2	3.85	3.76
3	—	3.89
Lysine		
1	3.68	—
2	3.84	—
3	3.77	—
Methionine		
1	3.69	3.75
2	3.84	3.78
3	3.76	3.87
4	—	3.70

In the second experiment, χ^2 tests of significance also showed significant differences among treatment effects, but, as indicated in table 2, and the orthogonal controls shown in table 3, the incidence of enteritis was significantly higher in the lots receiving methionine than the lots without it. Here, again, litter effects were highly significant.

Weaning weights (table 4) showed apparent effects of amino acid supplementation, whether by treatment or amino acid level, and litter effects, in both experiments.

Analysis of the weaning weight data (table 5) indicated highly significant differences among the treatments and between litters. In experiment 1, highly significant differences were found among the levels of lysine and the levels of methionine and a significant interaction between them. The difference between the levels of lysine and methionine were broken down into linear and quadratic effects, with the quadratic effect assuming greater importance in each instance. This curvilinear effect is apparent in table 4. In experiment 2, Duncan's multiple range test was used to separate the treatment differ-

TABLE 5

Analysis of variance of weaning weights

Source	d.f.	Mean square
Experiment 1		
Among treatments	8	1.0978[1]
Among levels of lysine (Ly)	2	1.6108[1]
Linear	1	0.9901[2]
Quadratic	1	2.1863[1]
Among levels of methionine (M)	2	1.4673[1]
Linear	1	0.5878
Quadratic	1	2.0227[1]
Interaction (Ly × M)	4	0.6518[2]
Between litters	1	6.8136[1]
Error	709	0.2060
Experiment 2		
Among treatments	3	1.0833[1]
Among litters	2	3.4068[1]
Error	906	0.2024

[1] Significant at the 1% level of probability.
[2] Significant at the 5% level of probability.

TABLE 6

Analysis of variance of feed conversion data

Source	d.f.	Mean square
Experiment 1		
Among treatments	8	2546.33
Among levels of lysine (Ly)	2	6600.00
Linear	1	3342.22
Quadratic	1	10,666.51[1]
Among levels of methionine (M)	2	28.97
Linear	1	29.07
Quadratic	1	37.91
Interaction (Ly × M)	4	2106.67
Between litters	1	4349.96
Error	116	2577.97
Experiment 2		
Among treatments	3	2390.38
Among litters	2	4888.21
Error	158	3072.64

[1] Significant at the 5% level of probability.

ences and it was found that treatment 3 (table 4), or 0.2% of methionine, resulted in weaning weights that were significantly greater than those of the three other treatments. There were no significant differences among the three remaining treatments. Differences between litter means were highly significant in both experiments, showing a linear increase in successive litters. Brinegar et al. ('50) and Bressani and Mertz ('58) showed that for swine and rats, respectively, minimum lysine requirements increased as dietary protein was increased. Since the stock ration used in these experiments contained approximately 22% of protein, as compared with a 14 to 16% protein ration used for postweaning stock, dry does and bucks, it is possible that amino acid requirements for young fryer rabbits fed high-protein rations are above those adequate for normal growth and development of older rabbits.

Analysis of feed conversion data revealed no significant treatment effects in either experiment (table 6). In experiment 1, however, when the sums of squares among the levels of lysine were broken down into linear and quadratic effects, there was a significant quadratic effect. The question then arises whether

the amino acid supplementation stimulates growth through increased efficiency of feed utilization or merely through increased appetite and feed consumption. Further studies involving controlled feed intakes are needed to answer this question.

SUMMARY

Supplementation of a basic rabbit ration with lysine and methionine gave conflicting results with respect to the incidence of enteritis, but significantly increased weaning weights of the young. No effects on feed conversion data were noted.

LITERATURE CITED

Acker, D. C., D. V. Catron and V. W. Hays 1959 Lysine and methionine supplementation of corn-soybean oil meal rations for pigs in drylot. J. Animal Sci., *18:* 1053.

Almquist, H. J. 1957 Proteins and Amino Acids in Animal Nutrition, ed. 4. U. S. Industrial Chemicals Company, New York.

Baron, H. 1958 Some effects of DL-methionine and glycocyamine on growth and nitrogen retention in rats. J. Nutrition, *64:* 229.

Bressani, R., and E. T. Mertz 1958 Relationship of protein level to the minimum lysine requirement of the rat. J. Nutrition, *65:* 481.

Brinegar, M. J., H. H. Williams, F. H. Ferris, J. K. Loosli and L. A. Maynard 1950 The lysine requirement for the growth of swine. Ibid., *42:* 129.

Casady, R. B., and E. M. Gildow 1959 Rabbit nutrition. Proc. Animal Care Panel, *9:* 9.

Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.

Germann, A. F. O., II, E. T. Mertz and W. M. Beeson 1958 Revaluation of the L-lysine requirements of the weanling pig. J. Animal Sci., *17:* 52.

Hagen, K. W., Jr. 1956 Infectious diseases of the rabbit. Department of Agriculture Yearbook, U.S.D.A., Washington, D. C., p. 559.

Hays, V. W., V. C. Speer, P. A. Hartman and D. V. Catron 1959 The effect of age and supplemental amino acids on the utilization of milk and soya protein by the young pig. J. Nutrition, *69:* 179.

Lund, E. E. 1951 Mortality among hutch-raised domestic rabbits, circ. 883, U. S. Department Agriculture, Washington, D. C.

National Research Council, Committee on Animal Nutrition 1954 Nutrient requirements for rabbits, pub. 331. National Academy of Sciences—National Research Council, Washington, D. C.

Squibb, R. L., M. K. Wyld, N. S. Scrimshaw and R. Bressani 1959 All-vegetable protein mixtures for human feeding. I. Use of rats and baby chicks for evaluating corn-based vegetable mixtures. J. Nutrition, *69:* 343.

Studies on the Availability of Amino Acids

I. EFFECT OF ALCOHOL TREATMENT OF CORN GLUTEN[1]

H. J. H. DE MUELENAERE,[2] M. L. CHEN[3] AND A. E. HARPER
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

Geiger and Hagerthy ('49) reported that a diet containing acid hydrolyzed-zein supplemented with amino acids supported a more rapid rate of growth of rats than a diet containing a proteolytic digest of zein supplemented with the same amino acids. Paper chromatography of a pepsin-trypsin digest of zein revealed three unidentifiable spots which were suggested to be enzyme-resistant peptides (Geiger et al., '52).

Deshpande and co-workers ('57) using a rat-growth method found that only 30% of the isoleucine in zein was biologically available. On the other hand the isoleucine in corn was noted to be approximately 90% available (De Muelenaere and Feldman, '60) as measured by a fecal analysis method. Since about 50% of the isoleucine in corn is in the zein, a lower value for the availability of isoleucine in corn would be expected on the basis of the results of Deshpande et al. ('57).

This raises the question of whether the low availability of the amino acids in isolated zein may be a result of changes that occur during the isolation of this protein. Although this possibility has been suggested by several workers (Albanese et al., '49; Geiger et al., '52) to our knowledge, no direct experimental evidence supporting it has been brought forward.

EXPERIMENTAL

In vitro studies

Materials. The study was carried out on defatted corn gluten (nitrogen content, 7.02%; zein content, 21.65%; fat content of original sample, 2.15%) and "reconstituted" corn gluten prepared by supplementing alcohol-extracted defatted corn gluten with a quantity of zein equal to the amount removed by alcohol treatment. Zein was extracted from corn gluten by the method of Mason and Palmer ('34). The defatted product which was not alcohol-extracted is referred to hereafter as untreated corn gluten.

Enzymatic digestion. Peptic digestion was carried out in duplicate on 2 gm of material suspended in 50 ml of distilled water. The pH was adjusted to 1.8, to this 40 mg of pepsin (1:10,000) was added, and the mixture was incubated for 24 hours at 37° to 38°C.

The pepsin digest was subsequently adjusted to pH 8.3 and 25 ml of a suspension, containing 0.15 gm of pancreatin was added. The resulting mixture was incubated for another 24-hour period. Toluene was added to each digestion mixture.

After incubation the enzymatic activity was stopped by heating for 10 minutes in a steam chamber. The insoluble residue was removed by centrifugation.

Acid hydrolysis. The acid hydrolysis of the corn gluten was carried out by heating 1 gm of sample in 250 ml of 2.5 N hydrochloric acid in an autoclave for 8 hours at 245°F. After concentration *in vacuo* the hydrolysate was made up to volume.

Determination of proteolytic activity. Proteolytic activity was determined by the direct formol titration (Brown, '23; Taylor, '57), and α-amino nitrogen was determined by the manometric micro-ninhydrin procedure (Van Slyke et al., '41). The latter provides an estimate of the free amino

Received for publication December 5, 1960.

[1] Published with the approval of the Director of Wisconsin Agricultural Experiment Station. This project was supported in part by a grant from the National Live Stock and Meat Board, Chicago.

[2] De Muelenaere, H. J. H., on leave of absence from the Natal Agricultural Research Institute, Natal, South Africa, is indebted to the South African Brewers' Institute for the award of the South African Breweries Overseas Research Fellowship.

[3] Fellow of the Williams-Waterman Research Corporation, New York.

acids released whereas the former should give a measure of free amino nitrogen in both peptides and free amino acids.

Chromatographic procedures for amino acid and peptide determinations. Aliquots of the enzymatic digest and the acid hydrolysate were reacted with fluorodinitrobenzene (FDNB) at pH 9.0. After removal of excess FDNB by ether extraction the reaction mixture was acidified to give an approximately 1 N HCl solution and extracted with five 15-ml portions of peroxide-free ether to remove the DNP-amino acids. The combined extracts were evaporated *in vacuo*, and the residue was taken up in acetone. The acetone solution was transferred quantitatively to a 17 × 22.5 inch Whatman no. 52 filter paper for two-dimensional chromatography. A mixture of toluene, chloroethanol, pyridine and ammonia was used as the solvent for the first dimension and concentrated phosphate buffer (1.5 M, pH 6.0) for the second (Levy, '54). After development, the chromatogram was dried, the spots were cut out and eluted overnight in 1% $NaHCO_3$. The optical density of each eluate was measured at 360 mμ using a Beckman DU spectrophotometer with the exception of DNP-proline which was measured at 385 mμ. The concentration of each DNP-amino acid was calculated from the appropriate standard curve prepared by plotting the optical density values for known amounts of the respective DNP-amino acid which had been subjected to the same chromatographic procedure as the unknown. Preliminary experiments proved that recoveries were 100 ± 15% for all amino acids except lysine.

The aqueous portion of the FDNB reaction mixture remaining after ether extraction was further extracted 5 times with ethyl acetate. The mixture was 85% neutralized with Na_2CO_3 and further extracted until no more yellow color could be removed. The combined ethyl acetate extracts were extracted three times with water, then dried *in vacuo* (Schroeder, '52). The residue was taken up in acetone, spotted and chromatographed as described for the ether soluble DNP-derivatives. All procedures were carried out in dimmed light.

Microbiological assay of amino acids. The amino acids in the enzymatic digests and acid hydrolysates were determined by microbiological assay using *Leuconostoc mesenteroides* for lysine, isoleucine, valine and *Streptococcus faecalis* for threonine (Barton-Wright, '52).

In vivo studies

Recovery of gastrointestinal contents. Twenty-four male rats of approximately 200 gm were trained for two weeks so they would consume about 4 gm of diet in a 30-minute period. The rats were divided into two sets of 12 animals each; one set received a diet containing corn gluten and the other, "reconstituted" corn gluten. Four rats from each set were killed at the following times: at the termination of the feeding period (zero time), one hour after and three hours after the end of the feeding period.

The stomach and small intestine were ligated and removed from the carcass. The contents of the stomach were washed out with water and the dry weight and total nitrogen content determined. The intestinal contents were washed into a centrifuge tube and made up to volume. The insoluble residue was separated by centrifugation. The supernatant solution was removed and 20% trichloroacetic acid (TCA) added to give a final TCA concentration of 5%. The tube was heated in a boiling water bath for 5 minutes and the TCA-precipitated material was removed by centrifugation. The nitrogen content of each fraction was determined.

The experimental diets contained 22% of protein from either corn gluten or "reconstituted" corn gluten; 5% of corn oil, 5% of salt mixture, vitamins and dextrin to make 100%.

Availability study. A growth method (Deshpande et al., '57), was used for the availability determinations. The basal diet contained an amino acid mixture (Calhoun et al., '60) that was complete except for the omission of the amino acid to be assessed; standard diets were prepared by adding to this, graded levels of the missing amino acid. Groups of 5 weanling male rats each were fed ad libitum for 14 days one of the standard diets or a test diet containing corn gluten or "reconsti-

tuted" corn gluten instead of the omitted amino acid. The amount of amino acid available to the animals fed each test diet could be determined by comparing their growth with the growth of groups receiving known amounts of the respective amino acid. From this and the amino acid content of the respective test rations the percentage availability was calculated.

RESULTS

In table 1 values for total soluble nitrogen and amino-nitrogen in the enzymatic digests are given as percentages of the respective values for the acid hydrolysates.

A marked difference in the degree of enzymatic digestion of untreated corn gluten and "reconstituted" corn gluten was evident in the samples subjected to the action of pepsin for 24 hours. Pepsin liberated roughly twice as much amino-nitrogen, as determined by either the formol titration or the Van Slyke method ('41), from the corn gluten as from the "reconstituted" sample; also, a greater portion of the corn gluten was solubilized. After pepsin-pancreatin digestion, the total soluble nitrogen and amino-nitrogen determined by formol titration remained considerably higher for the untreated corn gluten sample. The difference between amino-nitrogen values by the Van Slyke method decreased, the values being 10.9% for untreated corn gluten and 9.1% for the "reconstituted" sample.

The relative amounts of amino acids liberated by pepsin-pancreatin digestion of the samples are given in table 2. Threo-

TABLE 1

Total nitrogen and amino nitrogen in enzymatic digests of untreated and "reconstituted" corn gluten after pepsin and pepsin-pancreatin digestion

	Pepsin digestion		Pepsin-pancreatin digestion	
	Corn gluten	Reconstituted corn gluten	Corn gluten	Reconstituted corn gluten
Total soluble nitrogen in digest as % of nitrogen in acid hydrolysate	41.4	36.1	70.9	57.1
NH_2–N as % of NH_2–N in acid hydrolysate[1]	13.4	6.6	29.9	20.5
Free α–NH_2–N as % of α–NH_2–N[2] in acid hydrolysate[2]	3.9	1.5	10.9	9.1

[1] Formol titration.
[2] Van Slyke method ('41).

TABLE 2

Liberation of amino acids from untreated and "reconstituted" corn gluten by pepsin-pancreatin digestion as determined by chromatography and microbiological assay

Amino acids	Corn gluten		Reconstituted	
	Chromatographic	Microbiological[1]	Chromatographic	Microbiological
	(as percentage of amino acids in acid hydrolysate)			
Dicarboxylic acids	2.6		2.4	
Serine	6.2		6.3	
Threonine	6.4	64.9	5.8	50.8
Proline	3.0		1.9	
Alanine	8.3		8.9	
Glycine	3.3		—	
Valine	8.1	46.2	5.2	35.3
Leucine and isoleucine	28.5		27.3	
Isoleucine		39.4		33.6
Phenylalanine	14.6		17.6	
Tyrosine	10.2		10.9	
Lysine		57.7		51.8

[1] The microbiological assay was not carried out on the same digests as the chromatographic determination. The enzymatic digest was, however, prepared under similar conditions. No correction for enzyme is made in the microbiological assay.

nine, proline and valine in particular were liberated in larger amounts from the corn gluten than from the "reconstituted" sample.

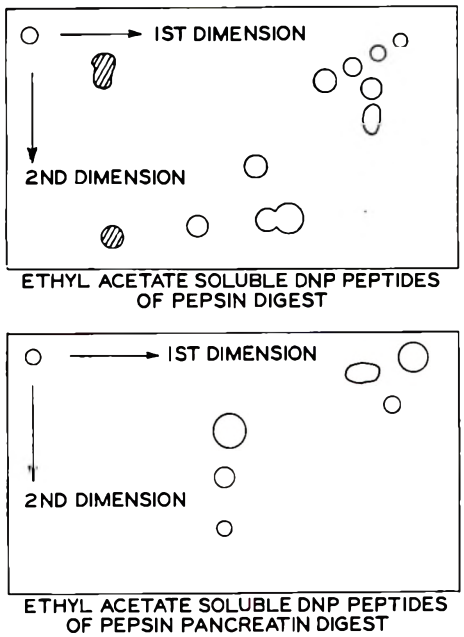

Fig. 1 Pattern of ethylacetate soluble DNP-peptides in the pepsin and pepsin-pancreatin digests.

The values for amino acids present in the digest as measured by microbiological assay were considerably higher than those obtained by the chromatographic method. The values for threonine, valine, isoleucine and lysine in the pepsin-pancreatin digest of corn gluten were all greater than those for the digest of "reconstituted" corn gluten, the differences between the respective values for threonine and valine being greatest.

Figure 1 represents the ethyl acetate-soluble DNP-peptide pattern of chromatograms of the pepsin and pepsin-pancreatin digests. Ten well-defined and two diffuse spots can be seen on the pepsin digest chromatogram. Six spots are found on the pepsin-pancreatin chromatogram.

The pattern of spots on the chromatograms of the corn gluten and "reconstituted" corn gluten digests were identical. The spots on the corn gluten chromatogram appeared to be somewhat denser, but no quantitative determination of the color intensity of the individual DNP-peptide spots was performed. A study of the amino acid compositions of the various DNP-peptides has been initiated but no complete results can be reported as yet.

In table 3 are shown the results of the *in vivo* digestion and absorption study.

TABLE 3

Nitrogen in insoluble, TCA-soluble and TCA-precipitable fractions of intestinal contents; dry residue of stomach; and ntirogen disappearance from gastrointestinal tract of rats fed untreated or "reconstituted" corn gluten

Ration	0 hour[1]	1 hour	3 hours
	Insoluble residue, mg nitrogen		
Corn gluten	6.40 ± 0.41[2]	9.84 ± 1.13	6.38 ± 0.48
Reconstituted	8.44 ± 0.39	24.87 ± 1.96	16.48 ± 1.64
	TCA-soluble nitrogen, mg		
Corn gluten	6.32 ± 0.34	8.24 ± 0.42	7.93 ± 1.0
Reconstituted	6.48 ± 0.26	8.20 ± 0.64	9.72 ± 0.35
	TCA-precipitated nitrogen, mg		
Corn gluten	2.03 ± 0.03	1.71 ± 0.12	2.26 ± 0.17
Reconstituted	2.55 ± 0.76	2.55 ± 0.14	2.78 ± 0.10
	Dry weight of residue recovered from stomach as % of total ingested		
Corn gluten	76.3 ± 0.69	59.1 ± 0.34	38.2 ± 4.13
Reconstituted	81.4 ± 0.64	55.5 ± 1.19	36.1 ± 1.02
	Disappearance of nitrogen from stomach and small intestine, mg		
Corn gluten	16.9 ± 0.87	33.5 ± 0.98	68.9 ± 1.32
Reconstituted	10.1 ± 2.32	22.6 ± 1.21	61.0 ± 1.59

[1] At end of 30-minute feeding period.
[2] Standard error of the mean.

The amount of intestinal residue was consistently higher in animals fed the "reconstituted" corn gluten diet. This and the greater rate of disappearance of nitrogen from the gastro-intestinal tract indicate that liberation and absorption of amino acids from the untreated corn gluten was higher than from the "reconstituted" sample.

In table 4 values for the availability of lysine, isoleucine, valine and threonine are given. Differences in availability of lysine and isoleucine from the two samples were small but the values for threonine and valine were considerably lower for the "reconsitituted" product than for the untreated corn gluten.

DISCUSSION

These results indicate that alcohol extraction reduces the availablity of some of the amino acids in corn gluten. The observation that lysine, which is highly susceptible to heat inactivation and which is not present in zein, was equally available in both products suggests that alcohol extraction affects mainly the zein fraction.

The values for availability given in table 4 must be considered only as relative values. The need for further investigation of the availability of amino acids in cereal products as determined by the growth method has been pointed out (Gupta et al., '58) and some of the factors that influence the results have been discussed (Calhoun et al., '60; De Muelenaere and Feldman, '60). A report of an investigation of the extent to which some of these factors limit the usefulness of the growth method is in preparation.[4]

Pepsin digestion liberated only a small percentage of the total amino acids as demonstrated by the Van Slyke α-amino nitrogen and free amino acid contents of the digests. In earlier *in vitro* studies, however, much higher availability values were reported (Denton and Elvehjem, '53; Bondi and Birk, '55). This latter was also true in the present study when the determination of amino acids was carried out by microbiological assay. The marked differences between liberation of amino acids as found by the chromatographic and microbiological assay must be attributed to the ability of the assay organism to utilize peptides (Klungsøyr et al., '51) and possibly to the growth promoting effect of certain peptides (Kihara and Snell, '55; Shankman et al., '60). In view of this it would appear that the microbiological assay is not a suitable analytical tool for assessing the free amino acid content of enzymatic digests.

In relation to the poor liberation of the dicarboxylic amino acids from both samples, the majority of the DNP-peptides on hydrolysis yielded glutamic and aspartic acids and several DNP-peptides contained glutamic acid as the NH_2-terminal amino acid.

The results of the *in vivo* and *in vitro* experiments indicating low availability of valine from "reconstituted" corn gluten are in good agreement and support the observation of Geiger et al. ('52) that considerable quantities of valine are excreted in the feces of rats fed a diet containing zein. The agreement between the results of the *in vitro* and *in vivo* studies on threonine is also good although the difference between the values for the two samples for the availability of this amino acid was smaller in the *in vitro* study (table 2).

Since pepsin and pepsin-pancreatin digestion of both untreated corn gluten and "reconstituted" corn gluten give identical DNP-peptide patterns, when chromatographed, it would appear that native and isolated zein are broken down enzymatically in a similar fashion, and hence that

[4] De Muelenaere, H. J. H., M. L. Chen and A. E. Harper, unpublished results.

TABLE 4

In vivo availability of lysine, isoleucine, valine and threonine from untreated and "reconstituted" corn gluten as determined by growth method

Protein source	Lysine	Isoleucine	Threonine	Valine
	%	%	%	%
Corn gluten	105.5	59.6	75.0	66.9
Reconstituted	103.9	55.7	59.3	43.1

no specific enzymatically resistant peptides are formed during the isolation process. A definite conclusion on this point, however, depends upon the assumption that either all of the DNP-peptides in the digest are ethyl acetate-soluble or that the ethyl acetate-insoluble DNP-peptides from the two samples would also show an identical pattern on chromatography.

The difference in the availability of isoleucine from corn gluten and "reconstituted" corn gluten is far less than anticipated at the outset of this work. This raises the question of whether the differences between the results of Deshpande et al. ('57) and de Muelenaere and Feldman ('60) cannot be traced to a basic difference in the techniques used. The first authors used a growth method, whereas the latter used a fecal analysis method. The growth method may well measure effects of amino acid balance as well as availability.

SUMMARY

An *in vitro* enzymatic digestion study of untreated corn gluten and "reconstituted" corn gluten (containing isolated zein) revealed that corn gluten containing native zein was digested at a faster rate than the "reconstituted" product.

Quantities of amino acids liberated after pepsin-pancreatin digestion were small compared with the quantities that were microbiologically available.

Identical DNP-peptide patterns were observed on chromatographing the digests of the respective samples.

In an *in vivo* study the ingested nitrogen disappeared more rapidly from the stomachs and intestines of animals fed the untreated corn gluten.

The values for availability of the threonine and valine in the "reconstituted" product were lower than those for untreated corn gluten. The values for the availability of isoleucine and lysine were not lowered by the treatment.

LITERATURE CITED

Albanese, A. A., S. E. Snyderman, M. Lein, E. M. Smetak and B. Vestal 1949 The biological value of corn and wheat protein in male infants with a note on the utilization of D-tryptophan. J. Nutrition, *38:* 215.

Barton-Wright, E. C. 1952 The microbiological assay of vitamin B complex and amino acids. Sir Isaac Pitman and Son Ltd., London.

Bondi, A., and Y. Birk 1955 The action of proteolytic enzymes on protein feeds. I. Liberation of terminal groups from peptic and pancreatic digests. J. Sci. Food Agr., *6:* 543.

Brown, J. H. 1923 The formol titration of bacterial media. J. Bact., *8:* 245.

Calhoun, W. K., F. N. Hepburn and W. B. Bradley 1960 The availability of lysine in wheat, flour, bread and gluten. J. Nutrition, *70:* 337.

De Muelenaere, H. J. H., and R. Feldman 1960 Availability of amino acids in maize. J. Nutrition, *72:* 447.

Denton, A. E., and C. A. Elvehjem 1953 Enzymatic liberation of amino acids from different proteins. J. Nutrition, *49:* 221.

Deshpande, P. D., A. E. Harper, M. Collins and C. A. Elvehjem 1957 Biological availability of isoleucine. Arch. Biochem. Biophys., *67:* 341.

Geiger, E., and E. B. Hagerthy 1949 Growth of rats on hydrolysed zein. Arch. Biochem. Biophys., *21:* 239.

Geiger, E., G. N. Courtney and L. E. Geiger 1952 The physiological availability of some essential amino acids present in zein, in acid and in the enzymatic hydrolysate of zein. Arch. Biochem. Biophys., *41:* 74.

Gupta, J. D., A. M. Dakroury, A. E. Harper and C. A. Elvehjem 1958 Biological availability of lysine. J. Nutrition. *64:* 259.

Kihara, H., and E. E. Snell 1955 Peptides and bacterial growth. VII. Relation to inhibition by thienylalanine, ethionine and canavanine. J. Biol. Chem., *212:* 83.

Klungsøyr, M., R. J. Sirny and C. A. Elvehjem 1951 Effect of incomplete hydrolysis on microbiological determination of amino acids. Ibid., *189:* 557.

Levy, A. L. 1954 A paper chromatographic method for the quantitative estimation of amino acids. Nature, *174:* 126.

Mason, I. D., and L. S. Palmer 1934 Preparation of white zein from yellow corn. J. Biol. Chem., *107:* 131.

Schroeder, W. A. 1952 Sequence of four amino acids at the amino end of the single polypeptide chain of lysozyme. J. Am. Chem. Soc., *74:* 5118.

Shankman, S., S. Higa, H. A. Florsheim, Y. Schvo and V. Gold 1960 Peptide studies. II. Growth promoting activity of peptides of L-leucine and L- and D-valine for lactic acid bacteria. Arch. Biochem. Biophys., *86:* 204.

Taylor, W. H. 1957 Formol titration an evaluation of its various modifications. Analyst, *82:* 488.

Van Slyke, D. D., R. T. Dillon, D. A. MacFadyen and P. Hamilton 1941 Gasometric determination of carboxyl groups in free amino acids. J. Biol. Chem., *141:* 627.

Excretion and Metabolism of Amino Acids in Rats[1]

P. A. HEDIN[2] AND M. O. SCHULTZE
Department of Agricultural Biochemistry, Institute of Agriculture, University of Minnesota, St. Paul, Minnesota

Several years ago it was reported from this laboratory (Schultze, '56) that rats could be maintained for successive generations with protein-free amino acid diets. The mixtures of amino acids then used contained several racemic components. In conjunction with that work, it seemed important to determine by criteria other than growth and reproductive performance to what extent the rat could utilize the amino acids contained in these diets. Earlier work on the utilization and metabolism of D-amino acids has been reviewed by Meister ('57). More recently Rechcigl et al. ('60) have reported that the D-amino acids can be used effectively as a source of nitrogen for the biosynthesis of nonessential amino acids by the rat.

The objective of the studies reported herein was to determine the nature and amounts of the major nitrogen-compounds in the urine of rats fed amino acid diets and to estimate the extent to which the D-isomer of amino acids can be utilized by this species.

EXPERIMENTAL

Diets. The diets AA_{15}, AA_{16} and AA_{19} contained 12.2, 18.3 and 24.4%, respectively, of a mixture of 16 amino acids (Schultze, '55) among which methionine, phenylalanine, threonine, tryptophan, valine, isoleucine, alanine and aspartic acid were racemic mixtures. The other components were 4% of a salt mixture, 3% of vegetable oil, a complete mixture of fat-soluble and water-soluble vitamins and variable amounts of sucrose as previously reported (Hallanger and Schultze, '56). To avoid contamination of the urine, these mixtures were administered by stomach tube to rats, twice daily, as 10 ml of an aqueous, homogenized suspension which contained 5.7 gm of the diet. The rats had access to water at all times. On this regimen the rats maintained constant weight and were in nitrogen balance for at least 4 weeks.

Animals. Male rats from our black strain, weighing about 300 gm, which had been reared with the diets mentioned previously, were housed in metabolism cages which permitted separate collection of excreta. Urine was collected in flasks containing a few drops of toluene. Quantitative collections of excreta were made from three to 5 rats receiving each diet for at least 14 days after the rats had become accustomed to forced feeding for about 10 days. Urine and feces were collected daily, pooled for the experimental period and frozen until the analyses were made. The pooled specimens represented for each diet the excreta from at least 50 rat-days.

Analytical procedures. Feces and urines were analyzed by a Kjeldahl procedure (Miller and Houghton, '45). Urine, in addition, was analyzed for ammonia and urea (Van Slyke and Cullen, '14), allantoin (Young and Conway, '42), creatine and creatinine (Folin, '14), and uric acid (Benedict and Franke, '22). Inasmuch as urine of rats is known to contain some protein, (Finlayson and Baumann, '56, '58; Rumsfeld, '56), it was hydrolyzed with an equal volume of concentrated hydrochloric acid in a sealed tube at 105°C for 48 hours. The hydrolyzed urine was adjusted to pH 9.0 and aerated to remove

Received for publication January 27, 1961.
[1] Paper no. 4555 Scientific Journal Series, Minnesota Agricultural Experiment Station. Reported in part at the 134th meetings of the American Chemical Society, September, 1958; (Abstracts, p. 48C). Financial support for part of this work from the Williams Waterman Fund of the Research Corporation is gratefully acknowledged.
[2] Present address: Quartermaster Food and Container Institute of the Armed Forces, 1819 W. Pershing Road, Chicago.

ammonia, prior to analysis for amino nitrogen (Frame et al., '43) which was corrected for the presence of allantoin. Urinary nitrogen calculated from the results of these analyses accounted for 98.3 to 102.5% of the total nitrogen determined by the Kjeldahl method.

Two-dimensional paper-chromatography (Levy and Chung, '53) of desalted (Carsten, '52) urine revealed the presence of at least 11 amino acids.

Tryptophan content of the urine was determined by the method of Dickman and Crockett ('56). The other amino acids in the urine (0.25 to 0.50 ml) were determined, before and after acid hydrolysis, by ion exchange separation with Dowex 50 × 4.2 (400-mesh) resin (Moore and Stein, '54a) and colorimetric analysis (Moore and Stein, '54b), procedures which were standardized with known mixtures of amino acids. The identity of the separated amino acids was established from the emergence pattern and verified by converting them, in aliquots of the eluates, into 2,4-dinitrophenyl derivatives (Levy, '54) which, in turn, were subjected, together with authentic specimens, to paper chromatography (Blackburn and Lowther, '51, Kent et al., '51). In this manner the excretion of small amounts of β-alanine was established. No evidence for the excretion of glucosamine and galactosamine could be obtained.

For determination of the excretion of D-amino acids, the amino acids in acid-hydrolyzed urine were measured, by the procedures described previously, before and after treatment with kidney D-amino acid oxidase. The enzyme preparation[3] was freed from ninhydrin-reactive contaminants by dialysis against 0.1 M pyrophosphate buffer, pH 8.5. The neutralized urine (0.25 ml) was buffered with pyrophosphate, pH 8.5, to 0.1 M concentration and treated at 38°C with 75 mg (before dialysis) of the dialyzed enzyme and 1.8 mg of flavin adenine dinucleotide[4] under a slow stream of oxygen for 8 hours; at this time an equal amount of enzyme was added and the reaction continued for another 8 hours. The mixture was then deproteinized with trichloroacetic acid and extracted with diethylether. Amino acids in the aqueous phase were separated with ion exchange resin and quantitatively determined as indicated above. Control experiments revealed that under these conditions L-amino acids were not affected whereas the loss of amino acids from racemic mixtures averaged 49.6%. Moreover, in a closed system, the oxygen consumption corresponded to the calculated value when D-alanine was used as substrate. A summary of the recoveries of known amounts of racemic amino acids achieved by these methods is presented in table 1.

TABLE 1

Recovery of amino acids[1]

	Amino acid oxidase treatment	
	Without treatment[2]	With treatment[3]
	% recovery	
DL-Aspartic acid	95.0	50.5
DL-Threonine	96.7	55.5
DL-Alanine	102.7	46.0
DL-Valine	94.6	50.5
DL-Methionine	98.0	48.5
DL-Leucine	98.0	50.0
DL-Phenylalanine	97.3	51.5

[1] From a mixture containing 200 μg of each of 14 amino acids.
[2] Average of three replications.
[3] Average of two replications.

Identification and determinination of 2-oxo-acids. From the oxo-acids in 20 ml of urine the 2,4-dinitrophenyl hydrazones were prepared by addition of 2 ml of a 0.2% solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid. After 20 minutes at 38°C, the mixture was cooled, extracted with ethyl acetate and this extract was extracted with 10% aqueous sodium carbonate solution. The alkaline extract was washed with ethyl acetate to remove excess reagent, acidified and re-extracted with ethyl acetate. The organic extract was concentrated to about 1 ml and a 0.05-ml aliquot was used for chromatographic separation, ascending, on Whatman no. 1 paper by 1-butanol, saturated with 3% aqueous ammonia.

For comparison with the unknown derivatives obtained from urine, reference compounds were prepared from authentic

[3] Nutritional Biochemicals Corporation, Cleveland.

[4] California Corporation for Biochemical Research, Los Angeles.

specimens of 2-oxo-acids,[5] including the 2,4-dinitrophenylhydrazones of the following compounds which are listed with their melting points and extinction coefficients ($E_{1cm}^{1\%}$ at 367.5 mμ): 2-oxo-3-methylvaleric acid 171°, 817; 2-oxo-4-methylvaleric acid, 162°, 749; 2-oxo-3-methylbutyric acid 196°, 788; 2-oxo-4-methylthiobutyric acid 146°, 752; 2-oxobutyric acid 126°, 838; pyruvic acid, 220°, 939; 2-oxoglutaric acid 240°, 928; oxalacetic acid 188°, 860; acetone 126°, 911. Quantitative determinations of the oxo compounds in urine were made by the method of Kun and Garcia-Hernandez ('57) using the previously cited extinction coefficients. When mixtures of 2,4-dinitrophenylhydrazones from urine were separated by paper chromatography and analyzed, the recovery was from 95 to 100%. All analyses were made at least in duplicate.

RESULTS AND DISCUSSION

Nitrogen compounds in urine. The rats were essentially in nitrogen balance under the conditions of this experiment. Fecal excretion of nitrogen was quite constant; it averaged about 45 mg per day, or 4 mg per gm of diet regardless of the nitrogen intake. This is about twice the amount which Mitchell ('34) reported for rats fed low-nitrogen diets by voluntary food intake. As much as 84.4% of the administered nitrogen was absorbed and hence an equal amount excreted in the urine.

The data summarized in table 2 demonstrate that (1) essentially all of the nitrogen in the urine was accounted for by the analytical methods; (2) as the nitrogen intake increased the absolute amounts of ammonia and amino compounds excreted in the urine also increased; and (3) from about 24 to 30% of the total nitrogen in the urine was excreted in the form of amino compounds, and an additional 10 to 12% as ammonia. The high proportion of nitrogen excreted in these compounds is in striking contrast to the composition of urine of rats fed a protein diet (Young and Cook, '55) and of other mammalian species (Albritton, '53) in which urea usually accounts for 80% or more of the total nitrogen. Under the conditions of these experiments the rats evidently excreted relatively large amounts of amino acids.

TABLE 2

Nitrogen distribution in rat urine

Nitrogen in	Ration fed		
	AA_{15}	AA_{16}	AA_{19}
	% of total urinary nitrogen[1]		
Urea	51.2	56.7	54.1
Allantoin	4.5	4.0	2.7
Uric acid	0.2	0.2	0.1
Amino groups[2]	30.3	26.8	23.7
Creatine	3.0	2.8	2.0
Creatinine	2.5	0.7	2.8
Ammonia	9.3	11.4	12.7
Above 7 components	101.0	102.6	98.1

[1] As determined by Kjeldahl method.

[2] As determined by the method of Frame et al. ('43).

Amino acids in urine. Inasmuch as the excretion of amino acids was greatest by rats which had the highest intake of amino acids, detailed results are presented for this group only (table 3). Identity of the amino acids (except for determination of the anomeric forms) which were separated by ion exchange resin was, with the exceptions noted later, verified by preparation of their 2,4-dinitrophenyl derivatives. These moved in all cases as single spots on paper chromatograms, with R_F values corresponding to those of the authentic specimens. No cystine, arginine, methionine or proline was detected in the urine. Small amounts of serine (1.4 and 2.4 mg per rat per day respectively) were found only in the urine of rats fed diets AA_{15} and AA_{16}. The component listed in table 3 as "after taurine" which was observed only after acid hydrolysis of the urines was not identified and ornithine was only tentatively identified from the emergence pattern during ion exchange separation. Acid hydrolysis of the urine increased the total amounts of detectable amino acids by about 14%, the increase being primarily due to glutamic acid, isoleucine, leucine and the unidentified "after taurine." Relatively large amounts of threonine, valine and isoleucine were excreted, partly, as will be shown later, in the form of D-isomers, and hence of dietary origin. Histidine was also excreted in

[5] Some of the 2-oxo-acids were generous gifts of Dr. Alton Meister whose assistance is gratefully acknowledged. Others were prepared in this laboratory from 2-amino acids which were treated with kidney amino acid oxidase.

TABLE 3

Amino acids in urine of rats[1]

Amino acid fed		Amino acid excreted		Excreted[2] / Fed × 100
		Before hydrolysis	After hydrolysis	
	mg/day	*mg/day*		
Taurine	0	29.1	32.0	
"After taurine"	0	0	10.8	
DL-Aspartic acid	147.8	4.2	8.0	5.4
DL-Threonine	182.4	68.2	63.9	35.0
L-Glutamic acid	558.6	15.5	21.5	3.8
Glycine	11.6	2.6	6.6	56.9
DL-Alanine	131.6	7.4	9.6	7.3
DL-Valine	328.4	94.0	90.9	27.7
DL-Methionine	82.0	0	0	
DL-Isoleucine	303.2	95.6	111.1	36.6
L-Leucine	288.4	0	7.5	2.6
β-Alanine	0	0	1.4	
DL-Phenylalanine	121.6	8.8[3]	13.5[3]	5.0
L-Tyrosine	150.4			
Ornithine	0	0	1.2	
L-Lysine hydrochloride	168.0	13.2[4]	12.7[4]	7.5
L-Histidine hydrochloride	79.2	15.8[4]	16.3[4]	20.6
DL-Tryptophan	42.2	3.3	—	7.8
L-Arginine hydrochloride	116.2	0	0	
L-Cystine	76.2	0	0	
Total	2787.8	357.7	407.0	

[1] Fed ration AA_{19} containing 24.4% of amino acids.
[2] Values obtained after hydrolysis.
[3] Calculated together because of failure to separate sharply.
[4] Calculated as hydrochloride.

TABLE 4

Urinary excretion of amino acids

	Diet		
	AA_{15}	AA_{16}	AA_{19}
Amino acids fed, mg/day/rat	1394	2089	2788
Amino acids excreted,[1] % of intake			
"Free" amino acids	13.2	8.8	12.7
"Bound" amino acids[2]	1.9	0.3	1.3
Total amino acids	15.1	9.1	14.0
D-Amino acids[3,4]	26.2[3]	10.5[3]	34.2[3]

[1] Includes only those amino acids present in the diet and taurine.
[2] Represents difference between the amino acids in urine before and after hydrolysis.
[3] Calculated as per cent of D-amino acids fed.
[4] Represents the difference between the amino acids in hydrolyzed urine before and after treatment with D-amino acid oxidase.

relatively large proportions, mostly in the free form, which is in accord with the suggestion of Evered ('56) that this amino acid has a high renal clearance. The other amino acids were excreted in much smaller quantities; their origin is not necessarily or solely dietary. The general pattern of amino acid excretion was similar in rats with all three different amino acid intakes, valine, isoleucine and threonine in each case being the most prominent components.

A summary of the total, free, "bound" and D-amino acid excretion observed with the three different diets is shown in table 4. The reason for the smaller percentage of free amino acids excreted by rats fed ration AA_{16}, which contained 18.3% of amino acids is not apparent; it may reflect their more efficient and more "normal"

TABLE 5

Preferential excretion of D-*amino acids*

Amino acid excreted	Isomer	Diet		
		AA_{15}	AA_{18}	AA_{19}
		% of intake[2]	*% of intake*[2]	*% of intake*[2]
Threonine	D-	62.3	9.1	41.0
	L-	14.5	26.8	33.8
Valine	D-	41.3	29.9	51.4
	L-	12.1	6.2	5.8
Isoleucine	D-	47.3	35.5	65.2
	L-	15.7	4.7	8.2

[1] The amounts fed per rat per day in diets AA_{15}, AA_{18} and AA_{19}, respectively, were 91.2, 136.8 and 182 mg DL-threonine; 164.2, 246.3 and 328.4 mg DL-valine; 151.6, 227.4 and 303.2 mg of DL-isoleucine.

[2] Of the corresponding isomer.

metabolic utilization, which is also suggested by the larger proportion of urea excreted by these rats (table 2).

It must be recognized that the administration of a whole day's ration in two portions does not correspond to the normal feeding habits of the rat and that temporary overloading of the organism with amino acids may have contributed to the extensive excretion of some amino acids and of ammonia. The relatively high concentration of ammonium compounds in the urine is in accord with the report by Cedrangolo et al.,[6] that after administration of D-amino acids the urinary excretion of ammonia increased more than that of urea.

The greatest proportion of "bound" amino acids (12.6% of the total) was found in the urine of rats fed the smallest amounts of amino acids, with diet AA_{15}. In man, Stein ('53) and Harrison and Harrison ('57) have observed urinary excretion of higher proportions of "bound" amino acids than reported here.

D-*Amino acids in the urine.* Treatment of the urines with D-amino acid oxidase revealed that, with one exception (threonine excreted by rats fed diet AA_{18}), the D-isomers accounted to a large extent for the relatively high concentrations of threonine, valine and isoleucine in the urines (table 5). The highest ratio D/L-isomer, namely 8.9, was observed with valine in the urine of rats fed 328 mg of a racemic mixture of this amino acid per day. With another diet which contained only 10 amino acids (Schultze, '55) the excretion of as much as 76.1 and 89.5% of the ingested D-isomers of valine and isoleucine respectively, has been observed in the urine of rats fed 319 mg of DL-valine and 228 mg of DL-isoleucine.[7] No satisfactory explanation is at hand for the great variability in the excretion of these D-amino acids under different dietary conditions. Evidence for the excretion of a relatively small proportion, 7.8% of the administered D-phenylalanine was obtained only in case of diet AA_{19} which furnished the largest amount of this compound, 60.8 mg per rat per day. The presence of D-aspartic acid, D-alanine or D-methionine could not be detected in any of the urine.

Excretion of 2-oxo-acids. The intermittent administration of relatively large amounts of amino acids to the rats used in these experiments and their subsequent rapid absorption and deamination could cause extensive production of 2-oxo-acids and their excretion in the urine. When the mixed 2,4-dinitrophenylhydrazones prepared from carbonyl compounds in urine were separated by paper chromatography, 9 components were observed. Among these, 8 compounds were identified and quantitatively determined (table 6). One unidentified component had an R_F value

[6] Cedrangalo, I. F., F. Salvatore, S. Papa and C. Saccone 1960 Urea and ammonium salts excretion after oral administration of ammonium salts or D-amino acids. Abstracts of Sectional papers, Fourth Internat. Congress Biochemistry, XV, p. 153, Pergamon Press, New York.

[7] Hedin, P. A. 1958 Products of metabolism of protein-free amino acid diets fed to rats. Ph.D. Thesis, University of Minnesota.

TABLE 6

Urinary excretion of carbonyl compounds

	Diet		
	AA_{15}	AA_{16}	AA_{19}
	mg/rat/day		
2-Oxo glutaric acid	21.15	20.30	13.20
Oxalacetic acid	0.90	0.99	0.63
Pyruvic acid	0.23	0.49	0.42
2-Oxo butyric acid	0.61	1.15	0.83
2-Oxo-4-methylvaleric acid	0.42	0.89	0.42
2-Oxo-4-methylthiobutyric acid	0.56	0.45	1.06
2-Oxo-3-methylbutyric acid	0.16	0.24	0.20
Acetone	0.08	0.15	0.21
Unidentified[1]	0.20	0.24	0.33
Total	24.31	24.90	17.30
Estimated excretion of dietary amino acids as 2-oxo-analogs, %	1.74	1.19	0.62

[1] Quantity estimated from mean absorption coefficients of other components.

of 0.88, slightly greater than that of the reference specimen of phenylpyruvic acid; this compound may have been formed from phenylalanine; it was not *p*-hydroxyphenylpyruvic acid acid, however.

The data in table 6 show that rats excreted the oxo-analogs of methionine, valine, leucine, glutamic acid, aspartic acid and alanine. For the latter three compounds, particularly, there is no assurance that these were produced by direct deamination of the dietary amino acids. The important point of the data in table 6 is the demonstration that despite relatively large intakes of amino acids (table 3) not more than a small fraction was excreted in the form of 2-oxo-analogs, even under the somewhat abnormal conditions of feeding used in these experiments. The rat can evidently metabolize not only the nitrogen of D-amino acids but also the deaminated carbon compounds. The derivatives of butyric acid and valeric acid listed in table 6 have previously been prepared by Waelsch and Miller ('42) from the urine of rats fed racemic mixtures of amino acids. Although no methionine was observed in the urine of the rats, its 2-oxo-analog was readily detected and measured. Isolation of the 2,4-dinitrophenylhydrazone of acetone from the urine of rats is no assurance that acetone was excreted; it may have been formed by decarboxylation of the acetoacetic acid derivative during isolation (El Hawary and Thompson, '53).

The early observations by Wohlgemuth ('05) and Dakin ('10) that the urine of animals contained predominantly the unnatural isomer following the administration of relatively large amounts of racemic tyrosine, suggested that the animal organism had a limited capacity to metabolize D-isomers of amino acids. Krebs ('35) showed subsequently, however, that kidney enzymes can deaminate *in vitro* the D-isomers of some amino acids more rapidly than the L-isomers. In the intact animal, however, the oxidation of the L-isomer may be more rapid (Hassan and Greenberg, '52) and more of the D-isomer may appear in the urine. The predominant excretion of some D-amino acids in the urine reflects the joint effects of differences in rates of metabolism, of absorption from the small intestine and, presumably also, from the renal tubules. Evidence for more rapid absorption of L-amino acids from the small intestine has been reported from several laboratories (Gibson and Wiseman, '51; Wiseman, '53; Agar et al., '56; Jervis and Smyth, 59a; Neil, '59). The presence of enantiomers in the lumen of the intestine, moreover, may affect the rates of absorption of the same and other amino acids (Jervis and Smyth, '59b). In the present experiments, restricted absorption of amino acids from the gut does not appear to have been a major limiting factor inasmuch as the fecal excretion of nitrogen was essentially the same regardless of the nitrogen intake. It is more likely

that a higher renal clearance of D-amino acids as reported for several species (Silber et al., '46; Crampton and Smyth, '53; Goodman, '56; Gartler and Tashian, '57) contributed to the presence of relatively large amounts of several D-amino acids in the urine which was observed in the present experiments.

SUMMARY

Protein-free diets containing 8 of 16 amino acids as racemic mixtures were administered to rats by stomach tube and the urine was analyzed for different nitrogen compounds, including D-isomers of amino acids and for 2-oxo-acids.

Under these conditions, the following observations were made:

1. Rats were in nitrogen balance and urinary nitrogen could be accounted for by urea, allantoin, uric acid, amino nitrogen, creatinine, creatine and ammonia.

2. Of the administered amino acids threonine, valine and isoleucine were excreted in the largest amounts.

3. As much as 13% of the amino acids in urine was in "bound" form, subject to acid hydrolysis.

4. As much as 62% of the administered D-threonine, 51% of D-valine and 65% of D-isoleucine was excreted in the urine.

5. The 2-oxo-acids in the urine accounted for not more than 1.75% of the administered amino acids.

LITERATURE CITED

Agar, W. T., F. J. Hird and G. S. Sidhu 1956 The absorption, transfer and uptake of amino acids by intestinal tissue. Biochem. biophys. acta, 22: 21.

Albritton, E. C. 1953 Editor, Standard Values in Nutrition and Metabolism. Handbook of Biological Data, part 2. McGregor and Warner Inc., Dayton, Ohio, page 202.

Benedict, S. R., and E. Franke 1922 A method for the direct determination of uric acid in urine. J. Biol. Chem., 53: 387.

Blackburn, S., and A. G. Lowther 1951 The separation of *N*-2:4-dinitrophenyl amino acids on paper chromatograms. Biochem. J., *48:* 126.

Carsten, M. E. 1952 A procedure for the ion exchange chromatography of the amino acids in urine. J. Am. Chem. Soc., *74:* 5954.

Crampton, R. F., and D. H. Smyth 1953 The excretion of the enantiomorphs of amino acids. J. Physiol., *122:* 1.

Dakin, H. D. 1910 The fate of inactive tyrosine in the animal body together with some observations upon the detection of tyrosine and its derivatives in the urine. The synthesis and probable mode of formation of Blend-Erdman's *para*-hydroxybenzyl-hydantoin. J. Biol. Chem., *10:* 25.

Dickman, S. R., and A. L. Crockett 1956 Reactions of xanthydrol. IV. Determination of tryptophan in blood plasma and in proteins. Ibid., *220:* 957.

El Hawary, M. F. S., and R. H. S. Thompson 1953 Separation and estimation of blood keto acids by paper chromatography. Biochem. J., *53:* 340.

Evered, D. F. 1956 Excretion of amino acids by the human: Quantitative study with ion exchange chromatography. Ibid., 62: 416.

Finlayson, J. S., and C. A. Baumann 1956 Role of urea in rat proteinuria. Am. J. Physiol., *184:* 469.

——— 1958 Mouse proteinuria. Ibid., *192:* 69.

Folin, O. 1914 On the determination of creatinine and creatine in urine. J. Biol. Chem., *17:* 469.

Frame, E. G., J. A. Russell and A. E. Wilhelmi 1943 The colorimetric estimation of amino nitrogen in blood. Ibid., *149:* 255.

Gartler, S. M., and R. E. Tashian 1957 Differential excretion of D-phenylalanine in man. Science, *126:* 75.

Gibson, Q. H., and G. Wiseman 1951 Selective absorption of stereo-isomers of amino-acids from loops of small intestine of the rat. Biochem. J., *48:* 426.

Goodman, R. M. 1956 Effect of W^v allele in the mouse on the differential excretion of the optical isomers of several amino acids. J. Exp. Zool., *132:* 189.

Hallanger, L. E., and M. O. Schultze 1956 Development of fatty livers during lactation of rats fed amino acid rations. J. Nutrition, 60: 25.

Harrison, H. E., and H. C. Harrison 1957 Amino aciduria in relation to deficiency diseases and kidney function. J. A. M. A., *164:* 1571.

Hassan, M. V., and D. M. Greenberg 1952 Distribution of label from metabolism of radioactive leucine, norleucine and norvaline in tissues, excreta and respiratory carbon dioxide. Arch. Biochem. Biophys., 39: 129.

Jervis, E. L., and D. H. Smyth 1959a The effect of concentrations of amino acids on their rate of absorption from the intestine. J. Physiol., *149:* 433.

——— 1959b Competition between enantiomorphs of amino acids during intestinal absorption. Ibid., *145:* 57.

Kent, P. W., G. Lawson and A. Senior 1951 Chromatographic separation of amino sugars and amino acids, using the *N*-(2:4-di-nitrophenyl) derivatives. Science *113:* 354.

Krebs, H. A. 1935 Metabolism of amino acids. II. Deamination of amino acids. Biochem. J., 29: 1620.

Kun, E., and M. Garcia-Hernandez 1957 Identification and quantitative determination of

keto acids by paper chromatography. Biochim. biophys. acta, 23: 181.

Levy, A. L. 1954 A paper chromatographic method for the quantitative estimation of amino acids. Nature, *174:* 126.

Levy, A. L., and D. Chung 1953 Two-dimensional chromatography of amino acids in buffered papers. Anal. Chem., 25: 396.

Meister, A. 1957 Biochemistry of the Amino Acids. Academic Press, Inc., New York.

Miller, L., and J. Houghton 1945 The micro-Kjeldahl determination of the nitrogen content of amino acids and proteins. J. Biol. Chem., *159:* 373.

Mitchell, H. H. 1934 The effect of the proportions of fat and carbohydrate in the diet upon the excretion of metabolic nitrogen in the feces. Ibid., *105:* 537.

Moore, S., and W. H. Stein 1954a Procedures for the chromatographic determination of amino acids on four percent cross-linked sulfonated polystyrene resins. Ibid., *211:* 893.

——— 1954b A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. Ibid., *211:* 907.

Neil, M. W. 1959 The absorption of cystine and cysteine from rat small intestine. Biochem., J. *71:* 118.

Rechcigl, M. Jr., J. K. Loosli and H. H. Williams 1960 D-Amino acids as source of non-specific nitrogen for growth of rats. Proc. Soc. Exp. Biol. Med., *104:* 448.

Rumsfeld, H. W. Jr. 1956 Role of dietary protein in normal rat proteinuria. Am. J. Physiol., *184:* 473.

Schultze, M. O. 1955 Concerning the alleged occurrence of an "animal protein factor" required for survival of young rats. II. Reproduction of rats fed protein-free amino acid rations. J. Nutrition, *55:* 559.

——— 1956 Reproduction of rats fed protein-free amino acid rations. Ibid., *60:* 35.

Silber, R. H., O. A. Seeler and E. E. Howe 1946 Urinary excretion of α-amino nitrogen following intravenous administration of amino-acid mixtures. J. Biol. Chem., *164:* 639.

Stein, W. 1953 A chromatographic investigation of the amino acid constituents of normal urine. Ibid., *201:* 45.

Van Slyke, D. D., and G. E. Cullen 1914 A permanent preparation of urease, and its use in the determination of urea. Ibid., *19:* 211.

Waelsch, H., and H. K. Miller 1942 The relation of keto acid excretion to amino acid metabolism. Ibid., *145:* 1.

Wiseman, G. 1953 Absorption of amino acids using an *in vitro* technique. J. Physiol., *120:* 63.

Wohlgemuth, J. 1905 Ueber das Verhalten stereoisomerer Substanzen im tierischen Organismns. II. Die inactiven Mono-amino säuren (Verläufige Mitteilung). Ber. deutsch. Chem. Gesellsch., *38:* 2064.

Young, D. R., and S. F. Cook 1955 Effect of environmental temperature and dietary protein on urinary nitrogen excretion of rats. Proc. Soc. Exp. Biol. Med., *89:* 482.

Young, E. G., and C. F. Conway 1942 On the estimation of allantoin by the Rimimi-Schryver reaction. J. Biol. Chem., *142:* 839.

Amino Acid Balance and Imbalance

VII. EFFECTS OF DIETARY ADDITIONS OF AMINO ACIDS ON FOOD INTAKE AND BLOOD UREA CONCENTRATION OF RATS FED LOW-PROTEIN DIETS CONTAINING FIBRIN[1]

U. S. KUMTA AND A. E. HARPER
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

A fall in food intake can usually be detected within a short time after an animal has been fed ad libitum a diet in which an amino acid imbalance has been created either by the addition of a relatively small amount of one or two amino acids (Harper, '58, '59; Harper and Kumta, '59; Kumta and Harper, '60) or by the addition of a larger amount of an amino acid mixture or unbalanced protein lacking one indispensable amino acid (Salmon, '58; Harper and Kumta, '59; Morrison and Harper, '60). This occurs both in weanling (Deshpande et al., '58) and in adult rats (Kumta et al., '58) within 24 hours after they have eaten a diet containing 6% of fibrin to which has been added from 0.5 to 1.0% of a mixture of the second most limiting amino acids, methionine and phenylalanine. In protein-depleted rats, a fall in the rate of ingestion of the diet containing additional methionine and phenylalanine can be detected within 4 hours (Harper and Kumta, '59) but food intake returns to normal a few hours later if the imbalance is corrected by a small supplement of the group of most limiting amino acids—leucine, isoleucine, valine and histidine.

The depression in food intake cannot be attributed to low palatability of the diet due to the addition of the amino acids causing the imbalance because rats trained to eat 12 gm of the basal diet consistently in two hours (single daily feeding), eat just as much of the imbalanced diet on the first day it is offered. On the second day, however, they eat less (Kumta et al., '58). Also, when protein-depleted rats are offered a diet containing amino acids in amounts that cause an imbalance, they eat at the same rate as the control group for about 4 hours, and only thereafter does their food intake fall. Correcting the imbalance by a further small supplement of amino acids prevents the fall in food intake. It therefore appears that the rate of food intake is influenced by some systemic effect of the unsatisfactory amino acid balance of the diet.

The experiments reported in this paper were undertaken in an effort to detect some metabolic or physiologic effect that occurred in rats within a short time after they had eaten a diet imbalanced in amino acids. The effect of an amino acid imbalance on the rate of stomach-emptying, on plasma urea concentration[2] and on the ability of the liver to synthesize tryptophan pyrrolase (Knox and Auerbach, '55) were studied. The effect of stimulating food intake by injecting the animals with insulin (MacKay et al., '40; Le Magnen, '56) was also determined.

EXPERIMENTAL

Male rats of the Holtzman strain were offered water ad libitum in all experiments and were fed either the basal diet or diets

Received for publication November 25, 1960.
[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Nutrition Foundation, Inc., New York, Monsanto Chemical Company, St. Louis, and from the Research Committee of the Graduate School from funds provided by Wisconsin Alumni Research Foundation.
[2] Kumta, U. S., and A. E. Harper 1960 Some further observations on amino acid imbalance. Federation Proc., *19:* 12 (abstract).

containing various additional amino acids under the different conditions to be described.

Diets

The basal diet used was essentially that described earlier (Kumta et al., '58). This contained (in per cent) fibrin, 6; salt mixture (Harper, '59), 5; corn oil, 5; vitamin supplements[3] (Harper, '59), 0.25; choline chloride, 0.15 and dextrin to make up 100. Fat-soluble vitamins A, D and E were included in the corn oil to provide the following concentrations per 100 gm of diet: A, 400 I. U.; D, 40 I. U.; and α-tocopherol, 10 mg. The protein-free diet was of the same composition as the basal diet except that the fibrin was replaced by dextrin. Additions of amino acids, as indicated in the tables, were compensated for by adjusting the percentage of dextrin.

Single daily feeding

Male rats weighing about 150 gm were trained to accept a single daily feeding according to the procedure outlined previously (Kumta et al., '58). During the training period the rats were fed the basal diet for only two hours each day. Only rats that gained the same amount of weight and which were consuming the same amount of food were used.

Stomach emptying. To determine the effect of an amino acid imbalance on the rate of stomach emptying, rats trained to accept a single daily feeding were fed: (1) the imbalanced diet prepared by adding 0.4% of DL-methionine and 0.6% of DL-phenylalanine to the basal diet, or (2) the basal diet made isonitrogenous with the imbalanced diet by the addition of 1% of L-glutamic acid. The rats were fed the imbalanced diet for two days consecutively before they were used for an experiment because food intake decreased only on the second day (Kumta et al., '58). On the day of an experiment the control and the experimental groups were fed the same amount of food; then at the various time intervals after the two-hour feeding period, as indicated in the results, 5 rats from each group were killed by ether anesthesia. The stomachs were removed and the contents washed with distilled water into tared aluminium dishes. The samples were then dried in an oven at 105° and weighed.

Effect on food intake. Several amino acid mixtures retard the growth rate of weanling rats (Kumta et al., '61); therefore 1% of each of these mixtures, and an equal quantity of others that do not, were added to the basal diet to permit comparisons of their effects on the food intake of rats trained to accept a single daily feeding. Comparisons were made over a period of 4 days. Each group consisted of 5 rats.

Protein depletion

Rats that weighed 120 to 130 gm were fed the protein-free diet for 4 days. Animals that lost approximately 15 to 20 gm were selected and separated into groups of 10 rats each. These were then fasted for 12 hours and were subsequently fed the various test diets for two hours. The amounts of food consumed were measured, and rats that ate the same amount were used for determining the effect of an amino acid imbalance on blood urea concentration and on the induction of tryptophan pyrrolase.

Plasma urea determinations. Groups of protein-depleted rats were anesthetized with ether at intervals after they had been allowed to eat for two hours, blood was withdrawn by heart puncture, and the plasma separated by centrifuging the heparinized blood samples. Protein-free filtrates were prepared using 10% sodium tungstate and 0.67 N H_2SO_4 (Folin and Wu, '19). Aliquots of the filtrate were used for the determination of urea by the colorimetric procedure of Archibald ('45). Values obtained by this method may be as much as 1.5 mg of urea per 100 ml of plasma above those obtained by the urease method.

Tryptophan pyrrolase. For the study of this inducible enzyme, the procedure used was that described by Knox and Auerbach ('55) with a few modifications. Four milliliters of a 0.33 M solution of L-tryptophan were injected into each rat. A separate group of rats was injected with 4 ml of a 0.9% saline solution. The protein-depleted rats were divided into several

[3] Some of the crystalline vitamins were kindly provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

groups (each consisting of 5 rats) and were fed: (1) a protein-free diet (2) the basal diet supplemented with 1% of L-glutamic acid, and (3) the basal diet supplemented with 0.4% of DL-methionine and 0.6% of DL-phenylalanine. A group of protein-depleted rats was also fed 2.5% of DL-ethionine. The rats were sacrificed 5 hours after the feeding period of two hours. The livers were removed and homogenized for two minutes with 7 to 9 volumes of cold 0.14 M KCl containing 0.0025 N NaOH. The homogenates were preincubated with or without 0.03 M L-tryptophan as the substrate for 5 minutes at 37°C. Following this, they were incubated for one hour with O_2 as the gas phase. A 15% solution of *meta*-phosphoric acid was used to stop the reaction and the amount of kynurenine formed was determined by measuring the absorption at 365 mμ in a Beckman D. U. spectrophotometer.

Insulin treatment

Male rats of 40 to 50 gm were fed the basal diet ad libitum for a preliminary period of 4 to 5 days to allow them to adapt to the diet and new environment. Rats that showed a progressive weight gain were selected and separated into groups of 5 that did not differ in average initial weight by more than 1 gm. These rats were offered the various experimental diets ad libitum and were weighed daily. Food consumption was also recorded. Insulin (protamine zinc insulin)[4] was injected subcutaneously to provide one unit per rat per day.

RESULTS

The results shown in figure 1 provided no evidence that an amino acid imbalance caused by adding methionine and phenylalanine to the 6% fibrin diet affected the rate of stomach-emptying of rats that had been fed a single large meal (11.8 gm). For the first 5 hours after the feeding period, the basal diet and the diet containing additional methionine and phenylalanine left the stomach at the same rate. The difference observed at 8 hours was not statistically significant. The stomach-emptying patterns resembled those described by Peraino et al. ('59) for rats fed 5 gm of a 15% casein diet but the rate

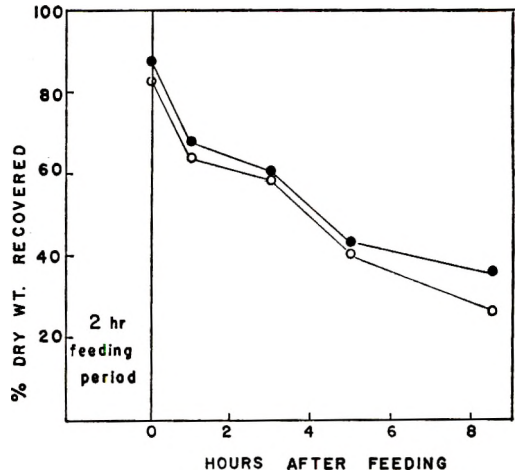

Fig. 1 Stomach emptying: Percentage of dry weight recovered at intervals after feeding diets for two hours. Average food consumption of each group was 11.8 gm; ○—○ basal diet containing 6% of fibrin + 1% L-glutamic acid; ●—● imbalanced diet containing 6% of fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine.

of emptying was somewhat slower in the present study possibly because of the much larger amount of diet fed.

Plasma urea concentrations were estimated at one, 5, 6 and 8 hours after feeding rats trained to accept a single daily feeding, equal amounts of either the basal diet or the diet containing additional methionine and phenylalanine. Average values for groups fed the diet containing methionine and phenylalanine were higher than those for the control groups at each time interval, with the greatest difference occurring three hours after the end of the feeding period (fig. 2). Similar results were obtained in an experiment in which protein-depleted rats were fed 5 gm of these diets. In the latter, plasma urea concentration rose to a maximum at three and one-half hours and returned to normal after 7 hours.

Plasma urea values for rats trained to accept a single daily feeding are generally much lower than values for protein-depleted rats (compare figure 2 and tables 1 and 2). The greatest difference has been observed with animals in the fasting state. The nature of the metabolic change responsible for this effect has not been studied.

[4] Abbott Laboratories, North Chicago, Illinois.

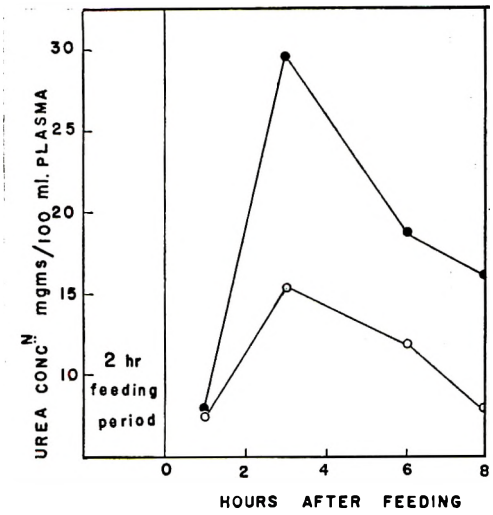

Fig. 2 Plasma urea concentrations at intervals after ingestion of 5 gm of diet in two hours; ●—● imbalanced diet containing 6% of fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine; ○—○ basal diet containing 6% of fibrin + 0.4% L-tyrosine + 0.25% DL-threonine (added to make the diets iso-nitrogenous).

To find out whether an elevated plasma urea concentration was characteristic of the imbalance caused by methionine and phenylalanine, various amino acid mixtures were tested for their effect on the food intake of rats trained for single daily feeding and, in separate experiments, plasma urea concentrations of protein-depleted rats fed the various diets were determined three hours after feeding them a single meal.

Amino acid mixtures that were known not to retard growth rate caused no elevation of plasma urea concentration nor any lowering of food intake (table 1). Plasma urea concentrations of the various groups ranged from 22 to 29 mg/100 ml of plasma with the value for the control group, which received additional glutamic acid, being 25.6 mg.

Among amino acid mixtures known to retard growth (table 2), only the combination of arginine and threonine caused an elevation of plasma urea concentration comparable to that observed for the group receiving methionine and phenylalanine, yet these amino acids, unlike the combination of methionine and phenylalanine, did not depress food intake within 4 days. A supplement of arginine and threonine de-

TABLE 1

Effects on plasma urea concentration and food intake of additions of amino acid mixtures that do not cause growth depressions in rats fed a diet containing 6% of fibrin as the source of protein

Amino acids added	Plasma urea concentration[1]	Daily food intake[2]			
		Day of experiment			
		1	2	3	4
	mg/100 ml	*gm*	*gm*	*gm*	*gm*
0.4% L-Cystine + 0.6% DL-phenylalanine	25.1 ± 2.0[3]				
0.4% L-Cystine + 0.6% DL-tyrosine	27.3 ± 2.8	10.5 ± 1.1[3]	10.2 ± 0.8[3]	10.8 ± 1.8[2]	10.7 ± 1.6[2]
0.4% DL-Methionine + 0.6% L-lysine	21.7 ± 3.0	9.21 ± 2.0	9.15 ± 1.8	9.2 ± 2.0	10.6 ± 1.5
0.4% DL-Methionine + 0.6% DL-tyrosine	23.2 ± 2.7				
0.4% DL-Methionine + 0.6% DL-threonine	29.1 ± 2.9				
0.4% DL-Threonine + 0.6% L-lysine	24.8 ± 3.3				
1% L-Glutamic acid (control)	25.6 ± 2.9	10.6 ± 1.6	11.0 ± 1.4	11.1 ± 1.8	11.5 ± 2.2

[1] Average values for groups of 6 protein-depleted rats three hours after ingestion of 5 to 6 gm of diet within a two-hour period.
[2] Average food consumption of a group of 5 adult rats trained for single daily feeding, determined in separate experiments.
[3] Standard error of the mean.

TABLE 2

Effects on plasma urea concentrations and food intakes of additions of amino acid mixtures that cause severe growth depressions in rats fed a diet containing 6% of fibrin as the source of protein

Amino acids added	Plasma urea concentration[1]	Daily food consumption[2]			
		Day of experiment			
		1	2	3	4
	mg/100 ml	*gm*	*gm*	*gm*	*gm*
0.4% DL-Methionine + 0.6% DL-phenylalanine	38.7 ± 2.8[3]	11.08 ± 1.8[3]	8.76 ± 1.5[3]	11.32 ± 1.8[3]	7.9 ± 1.0[3]
0.4% DL-Methionine + 0.6% L-leucine	24.6 ± 3.0	10.12 ± 2.8	6.52 ± 1.0	7.48 ± 1.5	8.2 ± 1.2
0.4% DL-Methionine + 0.6% L-arginine	25.6 ± 2.7	10.2 ± 1.6	8.28 ± 1.7	8.96 ± 1.6	9.56 ± 1.4
0.4% DL-Phenylalanine + 0.6% L-leucine	23.4 ± 3.6	9.5 ± 3.1	8.2 ± 1.9	7.6 ± 1.8	8.3 ± 1.6
0.4% DL-Phenylalanine + 0.6% L-arginine	29.01 ± 3.1	10.12 ± 1.4	10.08 ± 2.3	10.28 ± 2.0	10.08 ± 3.3
0.4% DL-Phenylalanine + 0.6% DL-threonine	22.3 ± 3.2	9.48 ± 1.9	8.48 ± 2.5	9.98 ± 2.7	10.28 ± 2.6
0.4% L-Arginine + 0.6% DL-threonine	39.7 ± 2.3	11.12 ± 1.4	10.76 ± 1.6	10.92 ± 2.4	11.12 ± 1.6
0.4% L-Arginine + 0.6% DL-tryptophan	24.8 ± 3.6	9.64 ± 0.6	6.84 ± 2.8	7.8 ± 2.2	8.0 ± 1.7
0.4% DL-Threonine + DL-tryptophan	22.6 ± 3.4	10.24 ± 1.3	7.0 ± 1.1	7.76 ± 1.1	8.6 ± 1.1
1% L-Glutamic acid (control)	23.2 ± 2.1	11.68 ± 1.2	11.52 ± 2.7	11.72 ± 1.4	10.56 ± 2.5

[1] Average values for groups of 6 protein-depleted rats three hours after ingestion of 5 to 6 gm of diet within a two-hour period.
[2] Average food consumption of a group of 5 adult rats trained for single daily feeding, determined in separate experiments.
[3] Standard error of the mean.

pressed both growth and food intake of weanling rats fed ad libitum after 5 to 8 days (Kumta et al., '61). Combinations of leucine with methionine or phenylalanine, which caused depressions of 56 to 75% in growth rate, (Kumta et al., '61) decreased food intake on the second day and the effect persisted on the third and 4th days. These amino acids, however, did not cause any rise in plasma urea concentration. Also, other amino acid mixtures which depressed food intake caused no elevation of plasma urea concentration such as that observed after feeding the diet containing methionine and phenylalanine.

Thus, among amino acid pairs known to cause growth depressions when added to the diet of weanling rats receiving 6% of fibrin, only the combinations of methionine and phenylalanine or arginine and threonine caused elevations in plasma urea concentration. Since growth depressions caused by these amino acids were prevented by a supplement of the most limiting amino acids, leucine, isoleucine, valine and histidine (Kumta et al., '61) plasma urea concentrations of rats receiving a supplement of these 4 amino acids with methionine and phenylalanine or arginine and threonine were determined. The results presented in table 3 show that no rise in plasma urea concentration occurred after the addition of the group of most limiting amino acids.

The ability of the liver to synthesize the adaptive enzyme, tryptophan pyrrolase, after rats had been fed equal amounts of the basal or the imbalanced diet is shown in table 4. In agreement with Knox and Auerbach ('55) a 10- to 12-fold response to injected tryptophan was obtained in 5 hours. The response was the same in rats fed a protein-free diet, the basal or the imbalanced diet, indicating that the reactions involved in the synthesis of this enzyme are not sensitive to the dietary balance of amino acids. Less enzyme was formed, however, when ethionine was added to the protein-free diet. Lee and Williams ('52) observed a similar inhibitory effect of ethionine.

The effects of injecting weanling rats fed either the basal or the imbalanced diet with insulin are shown in table 5.

TABLE 3

Effect of a supplement of the most limiting amino acids on plasma urea concentrations of rats fed a diet containing 6% of fibrin and methionine plus phenylalanine or arginine plus threonine

	Diets	Plasma urea concentration[1]
		mg/100 ml
1	6% Fibrin + 1% L-glutamic acid	23.2 ± 2.1[2]
2	6% Fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine	38.7 ± 2.8
3	Diet 2 + amino acid mixture[3]	26.8 ± 1.7
4	6% Fibrin + 0.4% DL-threonine + 0.6% L-arginine	39.7 ± 2.3
5	Diet 4 + amino acid mixture[3]	27.9 ± 1.8

[1] Average values for groups of 5 protein-depleted rats three hours after ingestion of 5 to 6 gm of diet within a two-hour feeding period.
[2] Standard error of the mean.
[3] The mixture of limiting amino acids contained: L-leucine, 0.1%; DL-isoleucine, 0.1%; DL-valine, 0.15%; and L-histidine·HCl, 0.05%.

TABLE 4

Tryptophan pyrrolase activity of liver 5 hours after injection of L-tryptophan into protein-depleted rats fed for two hours

	Diets	Tryptophan pyrrolase activity		
		Saline injected (1)	0.33 M tryptophan injected (2)	Difference (2 minus 1)
		μmoles of kynurenine/gm dry weight/hour		
1	Protein-free diet[1]	1.44	16.40	14.96
2	6% Fibrin + 1% L-glutamic acid	2.66	13.40	10.82
3	6% Fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine	1.84	14.30	12.46
4	Protein-free diet[2]	3.24	16.50	13.26
5	Protein-free diet + 2.5% DL-ethionine	3.16	8.44	5.28

[1] Protein free diet fed for 4 days.
[2] Protein free diet fed for 7 days.

TABLE 5

Effects of daily injections of insulin on the gain in weight and food consumption of rats fed on diets containing 6% of fibrin

	Diets	Injections		Gain in weight	Food intake
		Saline	Insulin[1]		
				gm/10 days	*gm/24 hours*
1	6% Fibrin	+	—	17.8 ± 1.8[2]	7.56 ± 0.8
2	6% Fibrin	—	+	17.7 ± 1.4	7.72 ± 1.8
3	6% Fibrin + 0.4 DL-methionine + 0.6% DL-phenylalanine	+	—	9.0 ± 2.2[3]	5.7 ± 1.6
4	6% Fibrin + 0.4% DL-methionine + 0.6% DL-phenylalanine	—	+	20.3 ± 0.8[3]	7.6 ± 1.6

[1] Protamin zinc insulin injected subcutaneously to provide 1 unit/rat/day.
[2] Standard error of the mean for 5 rats.
[3] The difference between these two groups is highly statistically significant.

Insulin did not stimulate the food intake of rats fed the basal diet but the food intake of rats fed the imbalanced diet containing additional methionine and phenylalanine was stimulated. The increased intake of the imbalanced diet did not retard growth, but on the contrary stimulated it significantly. In other experiments with an imbalance induced by an amino acid mixture lacking histidine, which causes a more severe depression in food intake, a number of rats died, presumably from insulin shock because they failed to eat enough food. The few rats that sur-

vived showed increased growth and food intake.

DISCUSSION

The almost immediate decrease in food intake of young rats fed a diet deficient in an amino acid (Rose, '38; Frazier et al., '47; Lepkovsky, '48) is generally attributed to a reduction in the need for all nutrients by animals that are not receiving enough balanced protein to permit a rapid rate of growth. A fall in the food intake of young rats fed a diet containing 6% of fibrin also occurs almost immediately when sufficient methionine and phenylalanine are added to cause an amino acid imbalance (Harper and Kumta, '59). Here, however, the quantity of balanced protein and the quantity of the most limiting amino acids in the diet remain unchanged. Food intake and, hence, growth of rats receiving such an imbalanced diet are stimulated if the concentrations of the most limiting amino acids or the quantity of balanced protein in the diet are increased. Animals fed an amino acid-deficient diet respond similarly to such supplements. This has led to the conclusion that an amino acid imbalance increases the need for the most limiting amino acid or acids in the diet, or in other words, that an amino acid imbalance increases the severity of an existing deficiency or may lead to the development of a deficiency where none existed before (Harper, '58).

Evidence that the efficiency of utilization of the most limiting amino acid in the diet is depressed has been presented (Kumta and Harper, '60). Such an effect would be anticipated if the rate of catabolism of amino acids were increased in some way owing to the imbalance. Evidence that the urinary excretion of amino acids increases owing to an amino acid imbalance has also been presented (Sauberlich and Salmon, '55; Salmon, '58) but the increased excretion was considered insufficient to account for the growth depression. The rise in the blood urea concentration of rats fed the diet in which an imbalance was caused by the addition of methionine and phenylalanine (fig. 1) and the prevention of the rise when the diet was further supplemented with the most limiting amino acids (table 3) suggests that this imbalance stimulates amino acid catabolism. However, a rise in blood urea concentration does not occur under similar conditions when certain other mixtures of amino acids that cause a reduction in food intake and a depression in growth rate are added to the diet (table 2). Thus, no general relationship between blood urea concentration and growth retardation or depression of food intake can be postulated. Whether this implies that the effects of the different amino acid mixtures are qualitatively different or only quantitatively different remains to be determined.

The possibility that the depression in the food intake of rats fed the diet containing methionine and phenylalanine is caused by a slowing of the rate of passage of food from the stomach would appear to be ruled out because no effect from the addition of methionine and phenylalanine on stomach-emptying was detected within 7 hours from the beginning of the feeding period (fig. 1), whereas a depression in food intake was detected within 4 hours (Harper and Kumta, '59).

Little attention has been paid in studies either *in vivo* or *in vitro* to the possibility that protein synthesis may be depressed if the amino acid pattern of the medium at the sites of synthetic activity deviates from the ideal pattern. Certainly the imbalance resulting from the addition of methionine and phenylalanine to a diet that is low in fibrin does not cause any inhibition of the synthesis of the enzyme tryptophan pyrrolase in response to an injection of tryptophan. The increase in enzyme activity probably represents little protein synthesis; hence the lack of an effect in this experiment, while suggestive, does not preclude the possibility that there may be conditions in which an amino acid imbalance can depress protein synthesis directly. The inhibition observed when the methionine antagonist, ethionine, is administered indicates that the synthesis of specific proteins can be inhibited *in vivo*.

The observation that rats fed the imbalanced diet gained as much as those fed the basal diet when food consumption was stimulated by the administration of insulin indicates that some basic mechanism regulating food intake is affected by an amino acid imbalance. The increased weight gain of the rats treated with insulin

is evidently due to the increased intake of the most limiting amino acids. As shown previously (Kumta and Harper, '60), the level of the most limiting amino acids in the diet is critical in demonstrating the growth-retarding effect of methionine and phenylalanine because this imbalance cannot be demonstrated if the fibrin content of the diet is increased much above 6%. Whether the increased growth rate as a result of insulin injections is due to the effect of insulin on amino acid transport (Kipnis and Noall, '58) or on the incorporation of amino acids into proteins (Manchester and Young, '58; Karner, '60; Doell, '60) or whether the effect is merely due to the stimulation of food intake because of the lowering of blood glucose (Morgan and Morgan, '40; Lawrence et al., '54) cannot be determined from these experiments. In any event, the appetite-depressing effect of the amino acid imbalance caused by adding methionine and phenylalanine to a diet containing 6% of fibrin is counterbalanced by the appetite-stimulating effect of insulin. The appetite-stimulating effect of insulin may be less effective when the imbalance is more severe.

SUMMARY

The low food intake of rats fed a diet containing 6% of fibrin supplemented with methionine and phenylalanine does not seem to be associated with a slower rate of stomach emptying. Feeding this imbalanced diet failed to reduce the ability of the rat to synthesize the enzyme tryptophan pyrrolase in response to a test dose of tryptophan.

The blood urea concentration of rats was greatly elevated three hours after the rats had ingested diets supplemented with mixtures of methionine and phenylalanine or arginine and threonine, both of which cause amino acid imbalance which result in depressions in growth and food intake. The rise in blood urea concentration was prevented by the further addition of the amino acids needed to correct the imbalance—leucine, isoleucine, valine and histidine.

Food intake was not depressed nor was blood urea concentration increased when mixtures of amino acids that do not cause growth depressions were included in the diet.

Several pairs of amino acids that caused depressions in growth and food intake did not cause a rise in blood urea concentration such as that observed in animals receiving methionine and phenylalanine.

Daily injections of insulin stimulated the growth and food intake of rats fed a diet containing 6% of fibrin supplemented with methionine and phenylalanine.

LITERATURE CITED

Archibald, R. M. 1945 Colorimetric determination of urea. J. Biol. Chem., *157:* 507.

Deshpande, P. D., A. E. Harper and C. A. Elvehjem 1958 Amino acid imbalance and nitrogen retention. Ibid., *230:* 335.

Doell, R. C. 1960 The effect of injected insulin on the amino acid incorporating system of rat liver. Biochem. Biophys. Acta, *39:* 237.

Folin, O., and H. Wu 1919 A system for blood analysis. J. Biol. Chem., *38:* 81.

Frazier, L. E., R. W. Wissler, C. H. Steffee, R. L. Woolridge and P. R. Cannon 1947 Studies in amino acid utilization. I. The dietary utilization of mixtures of purified amino acids in protein-depleted adult albino rats. J. Nutrition, *33:* 65.

Harper, A. E. 1958 Balance and imbalance of amino acids. Ann. N. Y. Acad. Sci., *69:* 1025.

——— 1959 Amino acid balance and imbalance. I. Dietary level of protein and amino acid imbalance. J. Nutrition, *68:* 405.

Harper, A. E., and U. S. Kumta 1959 Amino acid balance and protein requirement. Federation Proc., *18:* 1136.

Kipnis, D. M., and M. W. Noall 1958 Stimulation of amino acid transport by insulin in the isolated rat diaphragm. Biochem. Biophys. Acta, 28: 226.

Knox, W. E., and V. H. Auerbach 1955 The hormonal control of tryptophan peroxidase in the rat. J. Biol. Chem., *214:* 307.

Korner, A. 1960 The effect of administration of insulin to the hypophysectomized rat on the incorporation of amino acids into liver proteins *in vivo* and in a cell-free system. Biochem. J., *74:* 471.

Kumta, U. S., and A. E. Harper 1960 Amino acid balance and imbalance. III. Quantitative studies of imbalances in diets containing fibrin. J. Nutrition, *70:* 141.

Kumta, U. S., L. G. Elias and A. E. Harper 1961 Amino acid balance and imbalance. VI. Growth depressions from additions of amino acids to diets low in fibrin. Ibid., *73:* 229.

Kumta, U. S., A. E. Harper and C. A. Elvehjem 1958 Amino acid imbalance and nitrogen retention in adult rats. J. Biol. Chem., *233:* 1505.

Lawrence, R. T. B., J. M. Salter and C. H. Best 1954 The effect of insulin on nitrogen retention in the hypophysectomized rat. Brit. Med. J., 2: 437.

Lee, N. D., and R. H. Williams 1952 Inhibition of adaptive formation of tryptophan peroxidase in rats by ethionine. Biochem. biophys. acta, *9:* 698.

Le Magnen, J. 1956 Effets sur la prise alimentaire du rat blanc des administrations postprandiales d'insuline et le mechanisme des appetits caloriques. J. physiol., *48:* 789.

Lepkovsky, S. 1948 The physiological basis of voluntary food intake (appetite?) Adv. Food Res., *1:* 105.

MacKay, E. M., J. W. Callaway and R. H. Barnes 1940 Hyperalimentation in normal animals produced by protamine insulin. J. Nutrition, *20:* 59.

Manchester, K. L., and F. G. Young 1958 The effect of insulin on incorporation of amino acids into protein of normal rat diaphragm *in vitro.* Biochem. J., *70:* 353.

Morgan, C. T., and J. D. Morgan 1940 Studies in hunger. I. The effects of insulin upon rat's rate of eating. J. Genetic Psychol., *56:* 137.

Morrison, M. A., and A. E. Harper 1960 Amino acid balance and imbalance. IV. Specificity of threonine in producing an imbalance with diets deficient in niacin and tryptophan. J. Nutrition, *71:* 296.

Peraino, C., Q. R. Rogers, M. Yoshida, M. Chen and A. E. Harper 1959 Observations on protein digestion *in vivo.* II. Dietary factors affecting the rate of disappearance of casein from the gastrointestinal tract. Canad. J. Biochem. Physiol., *37:* 1475.

Rose, W. C. 1938 The nutritive significance of the amino acids. Physiol. Rev, *18:* 109.

Salmon, W. D. 1958 The significance of amino acid imbalance in nutrition. Am. J. Clin. Nutrition, *6:* 647.

Sauberlich, H. E., and W. D. Salmon 1955 Amino acid imbalance as related to tryptophan requirement of the rat. J. Biol. Chem., *214:* 463.

Tissue Lipid Fatty Acid Composition in Pyridoxine-Deficient Rats[1]

LEON SWELL, M. D. LAW, P. E. SCHOOLS, JR. AND C. R. TREADWELL
Veterans Administration Center, Martinsburg, West Virginia and Department of Biochemistry, School of Medicine, George Washington University, Washington, D. C.

A number of investigators have suggested that there is a relationship between pyridoxine and essential fatty acid metabolism. Witten and Holman ('52) reported that the conversion of linoleate to arachidonate was impaired in the essential fatty acid-pyridoxine deficient rat. The administration of linoleate to such doubly deficient animals produced an increased level of total body arachidonic acid, but the greatest deposition of that acid was noted when linoleate and pyridoxine were fed at the same time. Dam et al. ('58) have made similar observations in chicks. Goswami and Sadhu ('60) also reported that in pyridoxine-deficient rats there was a lower serum tetraenoic acid level compared with normal animals. Another indication of a relationship between pyridoxine and the essential fatty acids has been the observed similarity in the skin lesions of vitamin B_6- and essential fatty acid-deficient rats (Quackenbush et al., '42). In a recent study, however, Williams and Hincenbergs ('59) concluded that arachidonic acid and linoleic acid are not directly involved in the acrodynia of pyridoxine deficiency. Pyridoxine-deficient rats fed cottonseed oil or archidonic acid did not show any improvement in their dermal symptoms. Sinclair ('56) postulated that the essential fatty acids play an important role in the genesis of atherosclerosis and that pyridoxine might be indirectly involved since it is necessary for the conversion of linoleate to archidonate. In agreement with that hypothesis, Rinehart and Greenberg ('49) have shown that sclerotic lesions closely resembling those in man occur in pyridoxine-deficient monkeys. Recent studies from our laboratories (Swell et al., '60b) have demonstrated a correlation between the percentage of arachidonic acid in the serum cholesterol esters of several species and their susceptibility to atherosclerosis. Rat serum cholesterol esters have an exceedingly high content of archidonic acid (50%), whereas a susceptible species such as the rabbit has a very low level of that acid (1 to 2%). Because of the suggested relationship between the essential fatty acids and atherosclerosis and the possible role of pyridoxine in the synthesis of arachidonic acid, it seemed important to explore more fully the effect of pyridoxine deficiency on tissue lipids and fatty acids. Data are presented on the fatty acid distribution, as determined by gas-liquid chromatography, in the major tissue lipid fractions in normal and pyridoxine-deficient rats.

EXPERIMENTAL

Male rats (60 to 80 gm) of the Carworth strain were used. Each group (normal and pyridoxine-deficient) contained 28 animals. One group was fed a normal diet[2] consisting of vitamin-free casein, 27%; starch, 59%; vegetable oil, 10%; salt mixture (USP XIV), 4%; plus adequate amounts of the vitamins. The other group received the same diet with the omission of pyridoxine. Both groups of animals ate the same amount of food per day; the amount of food available to the normal group was governed by the intake of the experimental group. At the end of 40 days the rats fed the pyridoxine-deficient diet exhibited retarded growth and acrodynia. The average weight gain

Received for publication January 23, 1961.
[1] This work was supported in part by grants from U. S. Public Health Service (H-1897 and H-4374).
[2] Nutritional Biochemicals Corporation, Cleveland.

for the 40-day period in the control group was 142 gm and in the pyridoxine-deficient group, 66 gm. The animals were sacrificed (at the end of 40 days) and the following tissues removed: blood from the abdominal aorta, liver and adrenals. The average weight of the livers for the control group was 6.8 gm, and 6.2 gm for the pyridoxine-deficient group. The tissue lipids were extracted as described previously (Swell et al., '58) with the exception that following the initial extractions with 1:1 acetone-alcohol three additional extractions were carried out with 2:1 chloroform-methanol. Cholesterol esters, triglycerides and phospholipids were separated by chromatography on silicic acid according to Fillerup and Mead ('53) with several modifications. The silicic acid mixture consisted of 100 parts (100-mesh) Mallinckrodt silicic acid and 70 parts Hyflo Super-Cel. The mixture was heated for 24 hours at 120°C prior to use. Cholesterol esters were eluted with 1% ethyl ether in petroleum ether and triglycerides with 4% ethyl ether in petroleum ether. Next, the free cholesterol and minor lipid components were eluted with 25% ethyl ether in petroleum ether and discarded. The phospholipids were then eluted with 100% methanol. The isolated lipid fractions were weighed, interesterified in HCl methanol and the methyl esters sublimed according to the method of Stoffel et al. ('59). Gas-liquid chromatography was carried out as described earlier (Swell et al., '60a) with a succinate polyester of diethylene glycol as the stationary phase. Free and total cholesterol determinations on the original extracts, before chromatography, were carried out by the method of Sperry and Webb ('50). Triglyceride and phospholipid determinations were carried out by weighing the respective silicic acid column fractions. The determinations of *in vitro* corticoid secretion by the isolated rat adrenal was carried out as described by Eisenstein ('56). Total steroid hormone production was measured by the change in the absorption spectrum at 240 mμ. Steroid concentration was calculated from the absorption spectrum by the method of Allen ('50). Steroids with the 17,21-dihydroxy-20-ketone side chain were measured by the method of Porter and Silber ('50). Statistical analysis was carried out by the "t" test; values of $P < 0.01$ were considered to be significant.

RESULTS

Serum lipid fatty acids. The fatty acid composition of the different serum lipid fractions for both normal and pyridoxine-deficient rats are shown in table 1. The cholesterol ester fraction had the highest percentage of polyunsaturated fatty acids. In both normal and pyridoxine-deficient animals the polyunsaturated fatty acids of that fraction comprised 64 to 66% of the total fatty acids. Comparison of the cholesterol ester fraction in the two types of animals indicates that there was significantly less stearic acid ($P < 0.01$) and significantly more linoleic acid ($P < 0.01$) in the pyridoxine-deficient animals. The percentage of arachidonic acid was the same in both types of animals. The serum triglyceride fraction of the pyridoxine-deficient group had significantly less ($P < 0.01$) arachidonic acid than the normal group. Also, the deficient animals had more ($P < 0.01$) linoleic acid and less ($P < 0.01$) saturated fatty acids (palmitic and stearic) in that serum fraction. Comparison of the serum phospholipid fatty acid composition in both types of animals indicates that the only fatty acid showing a significant difference was linoleic acid; pyridoxine-deficient rats had significantly more ($P < 0.01$) of the acid. In table 3 are shown the levels of the major fatty acids in the several lipid fractions expressed as milligrams per 100 gm or ml. These values were derived from the percentage fatty acid composition (table 3) and the tissue lipid levels (table 6). Differences in the fatty acid levels paralleled the differences in the total fatty acids of the different serum lipid fractions. This was because there were no differences in the concentration of the different lipid fractions in the two groups.

Liver lipid fatty acids. Data obtained on the fatty acid composition of the major liver lipid fractions of normal and pyridoxine-deficient rats are shown in table 2. There were no significant differences in any of the fatty acids in the cholesterol esters between the two types of animals. The liver cholesterol ester fraction in both

TABLE 1

Effect of pyridoxine deficiency on the fatty acid spectrum of the serum lipid fractions

Fatty acid[1]		Cholesterol ester		Triglyceride		Phospholipid	
Chain length carbons	No. double bonds	Normal	Deficient	Normal	Deficient	Normal	Deficient
				% total fatty acids[2]			
6 to 12		2.0 ± 0.7[3]	1.3 ± 0.5	1.9 ± 0.6	0.9 ± 0.5	1.1 ± 0.4	0.9 ± 0.7
14	0	0.5 ± 0.2	0.5 ± 0.2	1.0 ± 0.4	0.8 ± 0.3	1.3 ± 0.9	0.9 ± 0.5
14	1	trace	trace	0.2 ± 0.1	0.2 ± 0.1	trace	trace
16	0	13.5 ± 1.9	12.6 ± 3.5	30.1 ± 4.0	23.0 ± 2.8	31.2 ± 3.9	30.5 ± 4.4
16	1	3.9 ± 1.1	2.5 ± 0.5	3.5 ± 0.8	2.1 ± 0.5	1.5 ± 0.4	1.2 ± 0.6
16	2	0.3 ± 0.1	0.3 ± 0.1	trace	0.2 ± 0.1	trace	0.2 ± 0.2
18	0	4.2 ± 1.3[4]	2.3 ± 0.8[4]	9.6 ± 1.9	5.3 ± 2.0	24.4 ± 4.0	20.3 ± 2.6
18	1	12.1 ± 3.4	14.6 ± 3.7	22.5 ± 2.0	27.5 ± 2.9	16.3 ± 3.1	13.6 ± 5.0
18	2	21.9 ± 3.5[4]	27.5 ± 1.9[4]	26.5 ± 4.1[4]	37.7 ± 3.6[4]	14.3 ± 3.6[4]	21.6 ± 5.5[4]
18	3	0.5 ± 0.1	0.7 ± 0.3	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.2
20	0	trace	trace	trace	0.2 ± 0.2	trace	trace
20	4	41.1 ± 8.4	37.7 ± 7.6	4.3 ± 2.4[4]	1.7 ± 0.7[4]	6.2 ± 3.4	6.4 ± 2.0
22[5]	—					3.2 ± 0.6	4.0 ± 0.7
Saturated		20.2	16.7	42.6[4]	30.2[4]	58.0	52.6
Mono-unsaturated		16.0	17.1	26.2	29.8	17.8	14.8
Polyunsaturated		63.8	66.2	31.2	40.0	24.2	32.6

[1] Represents major fatty acids determined; small amounts of others were also detected.

[2] Values represent the average of 14 determinations for the normal group (serum of two animals pooled per determination) and 8 determinations for the pyridoxine group (serum of 3 to 4 animals pooled per determination).

[3] Standard deviation.

[4] Significant difference $P < 0.01$.

[5] Represents fatty acids of 22 carbons and longer.

TABLE 2

Effect of pyridoxine deficiency on the fatty acid spectrum of the liver lipid fractions

Fatty acid[1]		Cholesterol ester		Triglyceride		Phospholipid	
Chain length carbons	No. double bonds	Normal	Deficient	Normal	Deficient	Normal	Deficient
				% total fatty acids[2]			
6 to 12		1.8 ± 0.9	0.8 ± 0.3	0.6 ± 0.4	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.2
14	0	0.8 ± 0.2	0.6 ± 0.3	0.8 ± 0.2	0.7 ± 0.2	0.3 ± 0.1	0.4 ± 0.2
14	1	0.2 ± 0.1	trace	trace	trace	trace	trace
16	0	30.0 ± 4.8	25.6 ± 5.1	31.4 ± 4.3	29.5 ± 4.5	30.1 ± 3.5	32.1 ± 4.8
16	1	3.2 ± 1.4	2.3 ± 0.7	3.5 ± 1.8	2.2 ± 0.5	1.3 ± 0.3	1.2 ± 0.4
18	0	19.7 ± 4.3	22.6 ± 4.1	4.5 ± 1.0[3]	7.6 ± 3.5[3]	27.4 ± 3.6	27.6 ± 5.3
18	1	23.2 ± 5.6	24.7 ± 4.3	23.8 ± 2.2	25.4 ± 2.4	9.8 ± 2.6[3]	13.8 ± 3.9[3]
18	2	15.8 ± 4.9	18.8 ± 5.0	28.8 ± 5.2	30.6 ± 5.5	15.7 ± 1.7	15.2 ± 4.3
18	3	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
20	0	0.3 ± 0.2	0.4 ± 0.3	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20	4	4.6 ± 1.2	3.7 ± 1.3	6.0 ± 1.4[3]	2.9 ± 1.5[3]	9.5 ± 2.7[3]	4.8 ± 2.9[3]
22[4]	—					5.0 ± 1.5	4.0 ± 1.3
Saturated		52.6	50.0	37.5	38.5	58.4	60.7
Mono-unsaturated		26.6	27.0	27.3	27.6	11.1	15.0
Polyunsaturated		20.8	23.0	35.2	33.9	30.5	24.3

[1] Represents major fatty acids determined; small amounts of others were also detected.

[2] Values represent the average of 14 determinations (livers of two animals pooled per determination).

[3] Significant difference $P < 0.01$.

[4] Represents fatty acids of 22 carbons and longer.

types of animals was characterized by a much lower level of polyunsaturated fatty acids (21 to 23%) than that fraction in the serum. Saturated acids comprised from 50 to 53% of the total fatty acids. This confirms our earlier finding (Swell et al., '60c) with respect to the dissimilarity in composition of the cholesterol ester fractions in the serum and liver of rats. In the liver triglyceride fraction there was significantly more ($P < 0.01$) stearic acid and significantly less ($P < 0.01$) arachidonic acid in the pyridoxine-deficient animals than in the normal animals. The arachidonic acid showed a decline of over 50%. Comparison of the phospholipid fatty acid composition in the two groups indicates that there was significantly more ($P < 0.01$) oleic acid and significantly less arachidonic acid in the pyridoxine-deficient animals. Here again, the decline in the arachidonic acid was substantial (approximately 50%). Table 3 shows the level of the different major fatty acids in the liver lipid fractions expressed as milligrams per 100 gm or ml. The most notable differences were in the phospholipid fatty acids. There was an absolute decline of the total fatty acids in that fraction of 47%. Although there were decreases in the levels of all the major fatty acids in that fraction, the most pronounced decrease was in the level of arachidonic acid which dropped from 217 to 49 mg/100 gm. The other major fatty acids decreased by approximately 50% in the pyridoxine-deficient animals. The cholesterol ester and triglyceride fatty acid levels paralleled the percentage total fatty acid as indicated in table 2.

Adrenal lipid fatty acids. The fatty acid composition of the several lipid fractions in both normal and deficient rats is shown in table 4. There were no differences between the two groups in the percentages of the polyunsaturated fatty acids in the several adrenal lipid fractions. The adrenal cholesterol ester fatty acids in both groups were characterized by a high level of those acids (approximately 47%).

Tissue lipid levels. In table 5 are shown the free and esterified cholesterol levels for serum, liver and adrenals of both types of animals. The free, ester and total serum cholesterol levels were the same in both normal and pyridoxine-deficient animals. Comparison of the liver cholesterol fractions shows that there was significantly less ($P < 0.01$) free cholesterol in the pyridoxine-deficient animals. The apparent increase of the liver cholesterol ester fraction in the pyridoxine-deficient animals was not significant. Due to the lower level of the free fraction in those animals, however, there was a considerable increase in the percentage of esterified cholesterol in the liver. No significant difference was observed in the adrenal cholesterol levels between the two groups.

Serum and liver levels for the three major lipid fractions are shown in table 6. The liver phospholipid level was markedly lower in the pyridoxine-deficient animals (17.3 mg/gm) than in the normal animals (32.1 mg/gm). The concentration of the cholesterol ester and triglyceride fractions was the same in both groups. There were no significant differences between the levels of the several lipid fractions in the serum of the two types of animals.

Steroid secretion. The steroid secretion of rat adrenal slices *in vitro* expressed as γ-corticoid produced per two hours is shown in table 7. Adrenal corticoid synthesis in both types of animals were found to be the same by the spectrophotometric method (total steroids) and the Porter-Silber procedure which measures 17,21-dihydroxy-20-ketone side chain steroids.

DISCUSSION

In the earlier studies (Witten and Holman, '52; Dam et al., '58; and Goswami and Sadhu, '60) the tetraenoic and other polyunsaturated fatty acids were determined by the alkali isomerization technique on the total tissue lipids. The findings of the present study indicate that when the fatty acid composition (gas-liquid chromatography) of the individual lipid fractions of the serum and liver are examined, only certain of the fractions show changes in polyunsaturated fatty acids as a result of pyridoxine deficiency. The cholesterol ester fraction of the serum, adrenal and liver did not show any significant decline in arachidonic acid; that fraction in the serum of the pyridoxine-deficient animals was high in arachidonic acid (38%). These observations support the

TABLE 3

Fatty acid levels in serum and liver lipid fractions of normal and pyridoxine-deficient rats

Fatty acid[1]		Cholesterol ester		Triglyceride		Phospholipid	
Chain length carbons	No. double bonds	Normal	Deficient	Normal	Deficient	Normal	Deficient
				Serum, mg/100 ml			
16	0	5.7	5.7	30.6	20.3	25.5	26.4
18	0	1.8	1.0	9.8	4.7	19.9	17.6
18	1	5.1	6.7	22.9	24.3	13.3	11.8
18	2	9.2	12.5	27.0	33.3	11.7	18.7
20	4	17.3	17.2	4.4	1.5	5.1	5.5
Total fatty acids		42.2	45.6	101.7	88.2	81.7	86.6
				Liver, mg/100 gm			
16	0	9.0	11.0	328	287	686	394
18	0	5.9	9.7	47	74	624	339
18	1	7.0	10.6	248	247	223	169
18	2	4.7	8.1	301	297	358	187
20	4	1.4	1.6	63	28	217	49
Total fatty acids		30.0	43.0	1044	972	2279	1228

[1] Represents only the major fatty acids; values derived from the percentage of the individual fatty acids (tables 1 and 2) × the total amount of cholesterol ester, triglyceride and phospholipid fatty acids (tables 5 and 6) per gram of liver or per milliliter of serum × 100. The factors for the percentage fatty acids in the lipid fractions were: cholesterol ester, 43; triglyceride, 90; and phospholipid, 71.

TABLE 4

Effect of pyridoxine deficiency on the fatty acid spectrum of the adrenal lipids

Fatty acid[1]		Cholesterol ester		Triglyceride		Phospholipid	
Chain length carbons	No. double bonds	Normal	Deficient	Normal	Deficient	Normal	Deficient
				% total fatty acids[2]			
6 to 12		1.4	1.2	1.0	0.3	0.6	0.9
14	0	2.3	1.6	1.8	1.6	1.2	1.6
14	1	0.3	0.2	trace	0.2	0.2	0.3
16	0	16.4	18.1	31.9	27.8	39.8	43.1
16	1	3.8	2.1	4.2	3.5	2.0	3.0
16	2	trace	trace	trace	trace	0.3	0.7
17	0	trace	trace	trace	trace	0.5	0.7
18	0	5.2	17.8	9.9	12.6	16.3	11.9
18	1	25.8	15.1	30.9	29.6	32.0	27.2
18	2	6.8	10.9	16.6	20.2	4.6	7.4
18	3	2.3	1.0	0.3	0.2	trace	trace
18	4	0.6	0.3				
20	0	0.5	0.4	trace	0.2	1.2	1.3
20	3	3.1	1.5	trace	trace	trace	trace
20	4	18.8	23.2	3.4	3.8	1.3	1.9
22	4	12.7	6.6				
Saturated		25.8	39.1	44.6	42.5	59.6	60.5
Mono-unsaturated		29.9	17.4	35.1	33.3	34.2	30.5
Polyunsaturated		44.3	43.5	20.3	24.2	6.2	9.0

[1] Represents major fatty acids determined; small amounts of others were also detected.
[2] The adrenals of 12 animals of each group were pooled for fatty acid analysis after removing aliquots for cholesterol determination (table 5).

TABLE 5

Tissue cholesterol levels in normal and pyridoxine-deficient rats

Group[1]	Tissue	Cholesterol			Ester Total
		Free	Ester	Total	
					%
Normal	serum, mg/100 ml	20 ± 4	62 ± 12	82 ± 15	75.6 ± 2.4
Pyridoxine-deficient	serum, mg/100 ml	21 ± 2	67 ± 4	88 ± 4	76.1 ± 2.5
Normal	liver, mg/100 gm	194 ± 28[3]	44 ± 17	238 ± 35	18.5 ± 6.1
Pyridoxine-deficient	liver, mg/100 gm	139 ± 24[3]	63 ± 24	202 ± 35	31.2 ± 9.2
Normal[2]	adrenals (2)	0.16 ± 0.04	1.22 ± 0.20	1.38 ± 0.26	88.4 ± 1.7
Pyridoxine-deficient[2]	adrenals (2)	0.16 ± 0.04	1.12 ± 0.18	1.28 ± 0.24	87.5 ± 2.0

[1] See footnotes to tables 1 and 2 for number of determinations and animals on serum and liver.
[2] Represents the average of 4 values; adrenals of three animals pooled per determination.
[3] Significant difference $P < 0.01$.

concept that the cholesterol ester fraction is important in the transport and metabolism of the essential fatty acids. The triglyceride fractions of the serum and liver showed a significant decline in arachidonic acid. It should be noted, however, that the triglyceride fraction had an initially low value of arachidonic acid. No change occurred in the level of arachidonic acid in the serum phospholipid fraction. There was a decline, however, in the amount of that acid in the phospholipid fraction of the liver, but this decrease, although substantial, was a reflection of a large absolute decrease in total liver phospholipid. The decrease in liver phospholipid may be the resultant of a deficiency of long-chain fatty acids, which would impair phospholipid synthesis. Thus, the data of the present study indicate that the ef-

TABLE 6

Serum and liver lipid levels of normal and pyridoxine-deficient rats

Group[1]	Cholesterol[2]	Triglyceride	Phospholipid	Total lipid
		Liver, mg/gm		
Normal	2.68 ± 0.40	11.6 ± 2.2	32.1 ± 3.8[3]	48.0 ± 6.9
Pyridoxine-deficient	2.45 ± 0.40	10.8 ± 4.7	17.3 ± 9.2[3]	33.3 ± 12.8
		Serum, mg/100 ml		
Normal	124 ± 23	113 ± 29	115 ± 22	
Pyridoxine-deficient	134 ± 10	120 ± 20	122 ± 16	

[1] See footnotes to tables 1 and 2 for number of animals and determinations.
[2] The total cholesterol value was obtained as the sum of the free cholesterol plus the esterified cholesterol calculated as cholesterol oleate (× 1.68).
[3] Significant difference $P < 0.01$.

TABLE 7

Steroid secretion by rat adrenal in vitro of normal and pyridoxine-deficient rats

Group[1]	Corticoid, γ/adrenal per two hours	
	Spectrophotometric method	Porter-Silber method
Normal	14.3 ± 2.7	5.1 ± 1.6
Pyridoxine-deficient	12.6 ± 2.6	5.1 ± 1.9

[1] Represents the average of 8 determinations per group (pool of two adrenals per determination).

fects of pyridoxine deficiency are localized in only certain lipid fractions, notably the triglyceride fractions of serum and liver and the phospholipid fraction of liver. The data also suggest that there is some impaired conversion of linoleate to arachidonate as evidenced by the lowered arachidonic acid level in some of the lipid fractions and the increased level of linoleate. The magnitude and the localization of this effect, however, suggests that pyridoxine may not act directly in the biosynthetic scheme from linoleate to arachidonate, but perhaps in some earlier metabolic reactions. Pyridoxine is known to be involved in transamination and decarboxylation reactions.

It has been reported (Goswami and Sadhu, '60) that pyridoxine deficiency produces a mild hypercholesterolemia and a drop in liver cholesterol which might be the result of an increased mobilization of liver cholesterol. In the present study pyridoxine deficiency had no significant effect on the serum cholesterol level. There was a significant decrease, however, in the liver free cholesterol and a slight increase in the ester cholesterol which was not significant. If the effect of pyridoxine deficiency on cholesterol levels were similar to that observed in essential fatty acid deficiency, one would expect to find an increase in liver cholesterol and a decrease in the serum cholesterol level (Alfin-Slater et al., '54).

The adrenal cholesterol ester fraction, as shown previously (Dailey et al., '60), is rich in polyunsaturated fatty acids (40 to 50%). It should be an excellent indicator of any changes which might occur in the adrenal polyunsaturated fatty acids due to pyridoxine deficiency. The results of the present study indicate that pyridoxine deficiency did not affect the amount of polyunsaturated fatty acids in the adrenal cholesterol ester fraction or the adrenal cholesterol level. Also, there was no effect on the adrenal triglyceride and phospholipid polyunsaturated fatty acids. It has been suggested (Sinclair, '58) that owing to the high content of polyunsaturated fatty acids in the adrenal there may be a relationship between those acids and adrenal cortical hormone synthesis. In support of this view Hayashida and Portman ('59) observed that the adrenals of essential fatty acid-deficient rats secrete smaller amounts of steroid hormones *in vitro*. In the present study, pyridoxine deficiency had no effect on the rate

of adrenal corticoid hormone production. At the same time, however, there was no change in the level of the adrenal polyunsaturated fatty acids. Therefore, this does not rule out the possible role which those acids might play in adrenal corticoid synthesis, but indicates that pyridoxine does not play a direct role in corticoid production.

SUMMARY

The influence of pyridoxine deficiency on the fatty acid composition (gas-liquid chromatography) of the cholesterol ester, triglyceride and phospholipid fractions of serum, liver and adrenal have been determined. Comparison of the tissue lipid fractions of both normal and pyridoxine-deficient rats indicated that only the triglyceride fraction of the serum and liver and the phospholipid fraction of the liver showed declines in the level of arachidonic acid and increases in linoleic acid. The cholesterol ester fatty acids of those tissues showed no significant change as a result of pyridoxine deficiency. Pyridoxine deficiency had no effect on the serum lipid levels, but there was a significant decline in the liver free cholesterol and a large decline in the liver phospholipid fraction (50%). Adrenal corticoid hormone synthesis was not impaired in the pyridoxine-deficient animal, nor was there any change in the level of the adrenal polyunsaturated fatty acids. The relationship of these findings to the role of pyridoxine in the biosynthesis of arachidonic acid and in the maintenance of tissue lipid and lipid fatty acids is discussed.

LITERATURE CITED

Alfin-Slater, R. B., L. Aftergood, A. F. Wells and H. J. Deuel, Jr. 1954 The effect of essential fatty acid deficiency on the distribution of endogenous cholesterol in the plasma and liver of the rat. Arch. Biochem. Biophys., 52: 180.

Allen, W. M. 1950 Simple method for analyzing complicated absorption curves, of use in colorimetric determination of urinary steroids. J. Clin. Endocrinol., *10:* 71.

Dailey, R. E., L. Swell, H. Field, Jr. and C. R. Treadwell 1960 Adrenal cholesterol ester fatty acid composition of different species. Proc. Soc. Exp. Biol. Med., *105:* 4.

Dam, H., G. Kristensen, G. K. Nielsen and E. Sondergaard 1958 Effect of pyridoxine deficiency on cholesterol and polyenoic fatty acids in chicks. Acta physiol. Scand., *44:* 67.

Eisenstein, A. B. 1956 Steroid secretion by the isolated rat adrenal. Proc. Soc. Exp. Biol. Med., *91:* 657.

Fillerup, D. L., and J. F. Mead 1953 Chromatographic separation of the plasma lipids. Ibid., 83: 574.

Goswami, A., and D. P. Sadhu 1960 Polyenoic acid in hypercholesterolaemia induced by pyridoxine deficiency in rats. Nature, *187:* 766.

Hayashida, T., and O. W. Portman 1959 Effects of essential fatty acid deficiency on rat adrenal composition and secretory activity. Am. J. Physiol., *197:* 893.

Porter, C. C., and R. H. Silber 1950 Quantitative color reaction for cortisone and related 17,21-dihydroxy-20-ketosteroids. J. Biol. Chem., *185:* 201.

Quackenbush, F. W., H. Steenbock, F. A. Kummerow and B. R. Platz 1942 Linoleic acid, pyridoxine and pantothenic acid in rat dermatitis. J. Nutrition, *24:* 225.

Rinehart, J. F., and L. D. Greenberg 1949 Arteriosclerotic lesions in pyridoxine-deficient monkeys. Am. J. Path., *25:* 481.

Sinclair, H. M. 1956 Deficiency of essential fatty acids and atherosclerosis, et cetera. Lancet, *1:* 381.

——— 1958 Essential fatty acids. 4th Internat. Conf. on Biochemical Problems of Lipids. Butterworths, London, p. 249.

Sperry, W. M., and M. Webb 1950 Revision of Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 97.

Stoffel, W., F. Chu and E. H. Ahrens, Jr. 1959 Analysis of long-chain fatty acids by gas-liquid chromatography. Micromethod for preparation of methyl esters. Anal. Chem., *31:* 307.

Swell, L., E. C. Trout, Jr., J. R. Hopper, H. Field, Jr. and C. R. Treadwell 1958 Mechanism of cholesterol absorption. I. Endogenous dilution and esterification of fed cholesterol-4-C^{14}. J. Biol. Chem., 232: 1.

Swell, L., H. Field, Jr., P. E. Schools, Jr. and C. R. Treadwell 1960a Fatty acid composition of tissue cholesterol esters in elderly humans with atherosclerosis. Proc. Soc. Exp. Biol. Med., *103:* 651.

Swell, L., H. Field, Jr. and C. R. Treadwell 1960b Correlation of arachidonic acid of serum cholesterol esters in different species with susceptibility to atherosclerosis. Ibid., *104:* 325.

Swell, L., M. D. Law, H. Field, Jr. and C. R. Treadwell 1960c Cholesterol ester fatty acids in serum and liver of normal and lymph-fistula rats. J. Biol. Chem., 235: 1960.

Williams, M. A., and I. Hincenbergs 1959 Methyl arachidonate supplementation of Vitamin B_6-deficient rats. Arch. Biochem. Biophys., *83:* 564.

Witten, P. W., and R. T. Holman 1952 Polyethenoid fatty acid metabolism. VI. Effect of pyridoxine on essential fatty acid conversions. Arch. Biochem. Biophys., *41:* 266.

Role of Enzymes in Metabolic Homeostasis[1]

II. DEPLETION AND RESTORATION OF AVIAN LIVER CARBOHYDRATE-METABOLIZING ENZYMES

GEORGE WEBER, GOURI BANERJEE, DAVID BIXLER AND JAMES ASHMORE

Departments of Pharmacology and Microbiology, Indiana University School of Medicine and School of Dentistry, Indianapolis, Indiana

Previous studies have established the important role in mammals of dietary factors which cause changes in the activity of certain hepatic enzymes. Adaptation to environmental alterations and the capacity of self-regulation are fundamental characteristics of living systems which result in the maintenance of a dynamic steady state called homeostasis. A basic requirement in maintaining homeostasis is the ability to alter the rate of metabolic reactions which underlie the various physiological processes. Since most of the metabolic processes are mediated through the activity of specific enzymes, information relating to regulation may be obtained by investigating the role of enzyme systems in the maintenance of homeostasis (Weber and MacDonald, '61).

Earlier investigations in mammalian systems demonstrated that the analysis of the biochemical sequence of events during starvation can be utilized to explore metabolic adaptations which the organism employs during the stress of sustaining blood sugar level through gluconeogenesis (Weber, '59). It was shown that prolonged starvation is reflected in a depletion of certain enzyme activities (Weber and Cantero, '58; Weber, '60). When starved animals were subjected to refeeding, however, a synchronized process of enzyme restoration occurred. Recently it was found that the events of enzyme depletion and restoration during fasting and refeeding can be interpreted in terms of enzyme breakdown and synthesis of the examined enzyme systems.[2]

Recent studies in this laboratory indicated that, with respect to adaptive response of certain hepatic enzymes, birds are more sensitive than rats to changes in composition of diet.[3] The present work describes the behavior of avian liver enzymes during processes of nutritional depletion and restoration. This investigation reveals that in birds, nutritional factors induce changes in hepatic enzymes essentially similar to those observed in mammalian systems.

MATERIALS AND METHODS

Animals and experimental procedures. Male Wistar rats weighing 180 to 220 gm and Leghorn chickens of 750 to 850 gm were obtained from local dealers. Rats were maintained with laboratory chow[4] and water ad libitum. Birds were maintained with a commercial feed.[5]

Preparation of liver homogenate and supernatant fluid. Animals were stunned, decapitated and bled. The preparation of homogenates and supernatant fluid (Weber and Cantero, '57a) and the determination of liver cellularity (Weber and Cantero, '57c) are described elsewhere.

Biochemical procedures. Enzymes were assayed in the supernatant fluid. Fructose-1,6-diphosphatase activity was determined as described by Weber and Cantero ('59).

Received for publication January 24, 1961.

[1] This investigation was supported in part by grants from the American Cancer Society (E254), National Cancer Institute, National Institutes of Health, U. S. Public Health Service (CY-5034) and U. S. Public Health Service (A-2701).

[2] Weber, G., G. Banerjee and S. B. Bronstein. Submitted for publication.

[3] Ashmore, J. Unpublished data.

[4] Purina Fox Chow, Ralston Purina Company, St. Louis.

[5] Farm Bureau Hi-Efficiency All Mash Layer No. 1, Indiana Farm Bureau Cooperative Association, Indianapolis.

Phosphohexoseisomerase activity was assayed according to Bruns and Hinsberg ('54), as modified by Glock et al. ('56). Phosphoglucomutase activity was assayed by the technique of Najjar ('48). Glucose-6-phosphate (G-6-P) dehydrogenase and 6-phosphogluconate dehydrogenase activities were followed by the method of Glock and McLean ('53). Lactic dehydrogenase activity was measured as described by Weber ('60). All enzyme assays were run at 37°C under linear kinetic conditions.

Expression of biochemical data. Enzyme activities were calculated in micromoles of substrate metabolized per hour per gram of wet weight of tissue at 37°C. Enzyme activities were expressed also per 100 gm of body weight. The various principles involved in the interpretation and expression of enzymatic alterations under physiological and pathological conditions were discussed previously (Freedland and Harper, '57; Weber and Cantero, '57b; Ashmore and Weber, '59; Weber, '59).

RESULTS AND DISCUSSION

Effect of 7-day fasting and one-day refeeding on liver and body weight. The effect of 7-day starvation and one-day refeeding on body weight, liver weight and liver/body weight ratio is expressed in percentage; the values of normal-fed chickens are taken as 100. The means and standard errors of the controls were the following: body weight, 808 ± 39; liver weight, 16.8± 0.5; liver/body × 100, 2.08 ± 0.07. During the starvation period the animals lost 58% of the initial body weight, liver weight decreased to 32% and liver/body ratio fell to 71% of the value of normal-fed chickens. On refeeding, body weight increased by 14% and liver weight by 32%. Liver/body weight ratio rose to 114% of the initial fed values. These percentage values are similar to results obtained in rats subjected to 6-day fasting (Weber, '60) and one-day refeeding (Weber and MacDonald, '61).

Comparison of avian and rodent liver carbohydrate-metabolizing enzymes. A comparison of the activities of avian and rodent hepatic carbohydrate-metabolizing enzymes is given in table 1. The enzyme pattern of the rats obtained in Indianapolis is essentially similar to that described previously in rats obtained and studied in Montreal. The only exception is the behavior of phosphoglucomutase which exhibited low activity in the Indianapolis rats.

The avian liver enzyme system pattern showed a close similarity to that noted in the Indianapolis rats with the exception of phosphohexoseisomerase which was twice as high in the chickens. Consequently, the most active enzyme in avian liver was phosphohexoseisomerase, whereas in rat liver it was lactic dehydrogenase.

The basic similarity in the pattern of liver enzyme activities is revealed when the activities are compared as percentage, taking phosphohexoseisomerase as 100. This parallelism suggests the possibility of similar carbohydrate metabolic mechanisms in avian and mammalian systems.

Effect of starvation and refeeding on avian hepatic carbohydrate-metabolizing activities. In table 2 is shown the be-

TABLE 1

Comparison of activities of liver carbohydrate-metabolizing enzymes in bird and rodent[1]

Enzymes[2]	Chicken	Rat (Indianapolis)	Rat (Montreal)[3]
Phosphohexoseisomerase	18300±1006(100)[4]	8166±337 (100)	10730±1733 (100)
Lactic dehydrogenase	10628±629(58)	14229±959 (174)	14982±857 (139)
Phosphoglucomutase	428±44(2.3)	467±27 (6)	6932±803 (65)
Fructose-1,6-diphosphatase	304.9±22(1.7)	382±17 (5)	302±23 (2.8)
6-Phosphogluconate dehydrogenase	127±3(0.7)	477±20 (6)	—
G-6-P dehydrogenase	5.8±1(0.03)	67.5±24 (0.8)	65±24 (0.6)

[1] Means and standard errors of 4 or more animals are given.

[2] Enzyme activities are expressed as μmoles of substrate metabolized/hour/gram wet weight of tissue at 37°C.

[3] Previously published data (Weber, '59).

[4] Enzyme activities are calculated as percentage, taking the value of phosphohexoseisomerase as 100.

TABLE 2

Depletion and restoration of avian hepatic enzymes[1]

	Fed, normal	Fasted 7 days	Refed 1 day	Increase	Restoration quotient[2]
Glucose-6-phosphate dehydrogenase	100	0	370	370	370
6-Phosphogluconate dehydrogenase	100	46	83	37	69
Fructose-1,6-diphosphatase	100	39	70	31	51
Phosphoglucomutase	100	48	93	45	87
Phosphohexoseisomerase	100	48	72	24	46
Lactic dehydrogenase	100	64	115	51	150

[1] Activity calculated per 100 gm body weight. Values are expressed in percentage, taking the normals as 100%.

[2] Restoration quotient $= \frac{\text{Enzyme increase on refeeding (\%)} \times 100}{\text{Enzyme activity lost during fasting (\%)}}$.

havior of liver enzyme systems in animals subjected to starvation and one-day refeeding. Seven-day starvation resulted in the disappearance of G-6-P dehydrogenase activity. Lactic dehydrogenase activity decreased to 64%, and all other enzymes decreased to 39 to 48% of the normal fed values. When animals were subjected to a one-day refeeding period G-6-P dehydrogenase activity rose to 370%, lactic dehydrogenase and phosphoglucomutase were restored to normal range, whereas the other enzymes increased to 70 to 83% of the normal fed values. These results draw attention to the fact already demonstrated in mammalian organisms that the enzyme systems involved in the metabolism of G-6-P exhibit widely differing behavior during processes involving enzyme depletion and restoration (Weber, '59; Weber and MacDonald, '61).

The differences in the extent of restoration of the avian liver enzyme activities are shown clearly when the percentage increase as a result of the refeeding period is tabulated (table 2). The marked increase in G-6-P dehydrogenase activity agrees with previous reports on the behavior of this enzyme in rodent liver (Tepperman, '58; Cohn and Joseph, '59; Weber and MacDonald, '61). The behavior of the other enzyme systems is also generally in line with that of liver carbohydrate-metabolizing enzyme systems in rats, with the exception of lactic dehydrogenase which is one of the slowest in returning in the rat, but is the second most rapid in the avian liver. When the restoration quotient is calculated for these enzyme activities, the different restoration rates of the various hepatic enzymes are brought out clearly. It is suggested that the different rates of restoration may be taken as an indication of the different synthetic rates of these enzyme systems which are manifested during the synchronized synthesis induced by the refeeding process (Weber and MacDonald, '61).

Current studies which showed that cortisone administration in hypophysectomized chickens resulted in a 200 to 300% increase in phosphohexoseisomerase and phosphoglucomutase activities in liver[6] suggest that the adrenal cortex may be involved, partly at least, in the mechanism responsible for re-establishment of previously depleted liver enzymatic activities.

The presented data show that avian hepatic enzyme activities are depleted and restored in varying degrees in fasted and refed animals. The behavior of enzyme systems in synchronized depletion and restoration shows remarkable similarity to that described in mammalian systems (Weber and MacDonald, '61).

SUMMARY

The dietary depletion and restoration of avian hepatic enzymes involved in carbohydrate metabolism were studied. Enzyme activities were depleted by 7-day fasting and restoration was induced by a one-day refeeding period.

In chickens 7-day fasting decreased glucose-6-phosphate dehydrogenase activity to zero, lactic dehydrogenase to 64%, and fructose-1, 6-diphosphatase, 6-phosphogluconate dehydrogenase, phosphoglucomutase and phosphohexoseisomerase activities to 39 to 48% of normal fed values.

[6] Weber, G., G. Banerjee, D. Bixler and J. Ashmore, unpublished results.

On refeeding, glucose-6-phosphate dehydrogenase increased to 300% and all other enzyme activities showed increases of 21 to 51%.

These results show that the various enzymes involved in glucose-6-phosphate metabolism show widely different behavior during nutritional depletion and restoration processes and do not all simultaneously change with the altering substrate level. Thus, the behavior of avian hepatic carbohydrate-metabolizing enzymes basically resembles that of mammals under the same conditions. These observations are well in line with the presented data which demonstrated that the general pattern of absolute activities of avian and rodent liver carbohydrate-metabolizing enzymes is also similar.

LITERATURE CITED

Ashmore, J., and G. Weber 1959 Biological chemistry of glucose-6-phosphatase. Vitamins and Hormones, *17:* 91.

Bruns, F., and K. Hinsberg 1954 Bestimmung und Eigenschaften der Phosphohexoseisomerase des Serums. Biochem. Ztschr., *325:* 532.

Cohn, C., and D. Joseph 1959 Effect of rate of ingestion of diet on hexosemonophosphate shunt activity. Am. J. Physiol., *197:* 1347.

Freedland, R. A., and A. E. Harper 1957 Metabolic adaptations in higher animals. I. Dietary effects on liver glucose-6-phosphatase. J. Biol. Chem., *228:* 743.

Glock, G. E., and P. McLean 1953 Further studies on the properties and assay of glucose-6-phosphate dehydrogenase of rat liver. Biochem. J., *55:* 400.

Glock, G. E., P. McLean and J. K. Whitehead 1956 Pathways of glucose catabolism in rat liver in alloxan diabetes and hyperthyroidism. Biochem. J., *63:* 520.

Najjar, V. A. 1948 The isolation and properties of phosphoglucomutase. J. Biol. Chem., *175:* 281.

Tepperman, H. M., and J. Tepperman 1958 The hexosemonophosphate shunt and adaptive hyperlipogenesis. Diabetes, *7:* 478.

Weber, G. 1959 Pathology of glucose-6-phosphate metabolism. A study in enzyme pathology. Rev. Canad. Biol., *18:* 245.

——— 1960 Effect of 6-day starvation on rat liver lactic dehydrogenase activity. J. Nutrition, *71:* 156.

Weber, G., and A. Cantero 1957a Glucose-6-phosphate utilization in hepatoma, regenerating and newborn rat liver, and in the liver of fed and fasted normal rats. Cancer Res., *17:* 995.

——— 1957b Effect of fasting on liver enzymes involved in glucose-6-phosphate utilization. Am. J. Physiol., *190:* 229.

——— 1957c Studies on hormonal factors influencing hepatic glucose-6-phosphatase. Endocrinol., *61:* 701.

——— 1958 Behavior of enzymes involved in glucose-6-phosphate utilization during 6-day fasting. Study of hepatic glucose-6-phosphatase, phosphohexoseisomerase and phosphoglucomutase. Exp. Cell Res., *14:* 596.

——— 1959 Fructose-1, 6-diphosphatase and lactic dehydrogenase activity in hepatoma and in control human and animal tissues. Cancer Res., *19:* 763.

Weber, G., and H. MacDonald 1961 Role of enzymes in metabolic homeostasis. I. Depletion and restoration of liver enzymes involved in glycolysis, glucogenesis and hexosemonophosphate shunt in normal and hypophysectomized rats. Exp. Cell Res., 22: 292.

Failure of Certain American Yeasts and of Selenium to Prevent Chronic Muscular Dystrophy in the Young Rat[1,2]

MARIANNE GOETTSCH
Department of Biochemistry and Nutrition, University of Puerto Rico, School of Medicine, San Juan, Puerto Rico

Alpha-tocopherol prevents the nutritional muscular dystrophy of rats deficient in vitamin E and the dietary necrotic liver degeneration of young rats receiving certain low-protein diets low in vitamin E. Muscular dystrophy is induced more readily in the presence of dietary cod liver oil and lard (Mattill, '47). Liver lesions develop in the absence of cod liver oil and independently of the lard content of isocaloric diets (Goettsch, '51).

The sulfur-containing amino acids, cystine and methionine, are effective in preventing hepatic necrosis in rats. Schwarz ('51) reported the isolation of a third factor, Factor 3, and the presence of selenium in the crystalline substance (Schwarz and Foltz, '57). Inorganic selenium is effective, not only in hepatic necrosis of the rat, but also in exudative diathesis of the chick, which occurs with certain vitamin E-low diets (Patterson et al., '57). Although selenium prevents liver necrosis in the rat and exudative diathesis in the chick, it is ineffective against sterility in the rat (Harris et al., '58), encephalomalacia in the chick (Dam et al., '57) and muscular dystrophy in the rabbit (Hove et al., '58). Schultze ('60) discusses the variable results obtained with lambs, calves, swine and chicks upon the role of selenium in muscular dystrophy.

Chronic muscular dystrophy, which has been reported in the adult vitamin E-deficient rat (Einarson and Ringsted, '38), appeared in young rats with certain vitamin E-low, low protein diets at the age of 6 weeks. The young rats had manifested acute muscular dystrophy at the end of the lactation period and recovered spontaneously. This acute muscular dystrophy appeared suddenly in 1954 after 7 years of failure in attempts to induce the disorder with the given technique (Goettsch and Pappenheimer, '41). Unknown factors may have been alterations in the vitamin E content of the commercial pellet ration or in the antioxidant activity of the lard used.

This report includes data on (1) breeding for the production of vitamin E-low young rats; (2) the relationship between the initial stores of vitamin E in the tissues and the incidence and time of onset of necrotic liver degeneration; (3) the occurrence of chronic muscular dystrophy in vitamin E-low young that do not die with liver lesions; (4) the prevention of hepatic necrosis by certain American yeasts and by selenium, confirming the results of Schwarz and Foltz ('57); (5) the failure of these yeasts and of selenium to prevent muscular dystrophy, a finding not in accord with the undocumented statement of Schwarz (Hove et al., '58).

EXPERIMENTAL

Diets. The diet for breeding rats in table 1 is a high-protein high-lard diet. Two kinds of lard were used in its preparation: a locally obtained lard that had obviously poor keeping qualities; stripped lard, low in vitamin E.[3] The locally obtained lard was used in the low-protein diets.

Received for publication January 24, 1961.
[1] This work was aided by a grant from the Dean's Fund of the University of Puerto Rico School of Medicine.
[2] Preliminary reports of part of this investigation were presented at the Third International Congress of Biochemists, Brussels, 1955 and the Third International Vitamin E Congress, Venice, 1955.
[3] Supplied by Distillation Products Industries, Rochester, New York.

TABLE 1

Composition of vitamin E-low diets

Constituents	Breeding diet	Low-protein diets[1]						
		Casein[2]	Yeast[3]					
	Parts	Parts	1%	2%	4%	8%	15%	30%
Crude casein[2]	26.6	8.3	—	—	—	—	—	—
Yeast	16.6[4]	—	1.0	2.0	4.0	8.0	15.0	30.0
Cornstarch	33.3	79.7	87.0	86.0	84.0	80.0	73.0	58.0
Salt mixture[5]	3.4	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Lard	18.3	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Cod liver oil	1.8	2.0	2.0	2.0	2.0	2.0	2.0	2.0

[1] Synthetic vitamins added as supplement to each kilogram of low-protein (casein) diet: (in milligrams) thiamine·HCl, 5; pyridoxine·HCl, 5; riboflavin, 10; niacin, 20; vitamin K, 5; PABA, 10; Ca pantothenate, 50; folic acid, 4; and biotin, 0.4. Half of the amount was added to the low-protein yeast diets.

[2] Casein, Grade B-1-F Crude, Casein Company of America, New York.

[3] Types of yeast used in the low-protein diets were as follows: Fleischmann, type 2019; food yeast (*Torula utilis*, no. 3, locally grown); Anheuser Busch, Strains G and K_2; St. Louis Corporation, regular non-debittered dried brewers'; Jos. Schlitz, Strains Fe-2 and Fo-2. The American yeasts were obtained through the courtesy of Dr. Schwarz and of Miss Singruen of the Brewers' Yeast Council, Inc. We are indebted to them and the yeast companies for their cooperation.

[4] Fleischmann, type 2019.

[5] Salt mixture no. 2, USP, Nutritional Biochemicals Corporation, Cleveland.

The low-protein diets contained either crude casein or yeast as the source of protein. The 8.3% casein diet and the 15 or 30% yeast diets (Fleischmann, type 2019, or *Torula utilis*, number 3, locally grown) have given consistent results under the given experimental conditions (Goettsch, '51). The other known strains of American yeasts, described in a footnote of table 1, were used in concentrations of from 1 to 15%. The 8.3% casein and 30% Torula diets were supplemented with 0.5 ppm of selenium as sodium selenite.

Procedure. Wistar rats, acclimatized to the tropical marine conditions of Puerto Rico, were reared with a commercial pellet ration and bred at estrus after 90 days of age. The vitamin E-low diet for breeding rats was fed from the beginning of gestation until the end of lactation, in order to reduce the initial stores of vitamin E in the young rats (Goettsch, '51). Vitamin E-low young rats that survived the period of lactation were given the low-protein diets from the 21st day of life or from the day that the paretic symptoms of acute muscular dystrophy disappeared. The mean initial body weight was 35 (25–50) gm. Surviving animals were sacrificed after 90 days with the diet. Liver and muscle changes were confirmed by microscopic examination of the tissues which did not present gross lesions.

RESULTS AND DISCUSSION

Fertility. Fertility data in table 2 show the extent of biological variation among groups of rats. Since there was no significant difference in fertility performance between the rats receiving the vitamin E-low lard and those fed the lard of poor keeping qualities, the data were combined. The following observations were made upon 96 rats, reared with the commercial pellet ration and given the vitamin E-low breeding diets from the beginning of gestation: 46 bore litters of living young; in 17 litters the young were dead at the time that the litters were examined; three rats underwent typical resorption gestations; 30 died during parturition after a prolonged gestation period with symptoms of eclampsia such as those described by Stamler ('59) in rats given small amounts of vitamin E. The fetuses were large and appeared well developed.

The administration of 10 mg of α-tocopheryl acetate to 20 rats at the beginning of gestation induced normal fertility.

Among three groups of rats reared and bred with the commercial pellet ration, there were two rats with litters of dead

TABLE 2

Fertility of rats reared with a commercial pellet ration and given certain vitamin E-low diets during gestation, with and without the addition of α-tocopherol

Year	Gestation diet			No. of rats	Number with			
	Pellet	Vitamin E-low breeding diet			Litters of young		Resorptions	Death during parturition
		Kind of lard			Living	Dead		
		Poor-quality	Vitamin E-low					
1955		+		31	22	5	0	4
1956		+		10	5	1	0	4
1956			+	15	7	1	0	7
1958		+		28	8	9	2	9
1958			+	12	4	1	1	6
Total				96	46	17	3	30
10 mg α-tocopheryl acetate at beginning of gestation								
1958		+		9	9	0	0	0
1958			+	11	11	0	0	0
Total				20	20	0	0	0
1956	+			10	10	0	0	0
1957	+			14	13	0	0	1
1958	+			17	13	2	0	2
Total				41	36	2	0	3

TABLE 3

Incidence of muscular dystrophy among nurslings of rats given vitamin E-low diets from the beginning of gestation, with and without the addition of α-tocopherol

Year	Gestation and lactation diet			No. of litters with living young	Young rats			
	Pellet	Vitamin E-low breeding diet			No. living		With muscular dystrophy	
		Kind of lard			At birth	At 21 days	Number	Number dying by 28th day
		Poor-quality	Vitamin E-low					
1955		+		22	181	148	99	6
1956		+		5	52	50	20	4
1956			+	7	66	60	60	26
1958		+		8	69	47	55	26
1958			+	4	35	32	17	0
Total				46	403	337	251	62
10 mg α-tocopheryl acetate at beginning of gestation								
1958		+		9	83	69	13	0
1958			+	11	94	77	4	2
Total				20	177	146	17	2
1956	+			10	119	116	0	
1957	+			13	127	117	0	
1958	+			13	147	114	2	2
Total				36	393	347	2	2

young and three rats that died during parturition with findings similar to those observed with the vitamin E-low diets. Apparently the commercial pellet ration did not contain sufficient vitamin E for normal reproduction in all rats.

Acute muscular dystrophy during lactation. The incidence of muscular dystrophy among young rats of females receiving the vitamin E-low breeding diets was not influenced by the type of lard used. The data were combined in table 3. "Pa-

TABLE 4

Incidence of necrotic liver degeneration and muscular dystrophy among young rats with vitamin E-low low-protein diets, with and without selenium

Diet	No. of rats	Rats with necrotic liver			Rats with normal liver			
			Days fed diet			With muscular dystrophy		
		No.	Mean	Range	No.	No.	Days fed diet	
							Mean	Range
Young reared with pellets								
8.3% casein	28	10	34	16–35	18	0	90[1]	
30% Torula	11	10	29	21–71	1	0	49	
Vitamin E-low young								
8.3% casein	56	54	10	5–26	2	2	32	13–50
30% Torula	12	11	10	3–32	1	0	36	
15% Torula	12	12	8	4–13	0			
15% Fleischmann	12	11	9	6–15	1	1	18	
15% A. B., Strain G	12	6	9	4–13	6	4	57	17–90[1]
15% J. S., Fe-2	12	1	17		11	11	58	30–90[1]
15% J. S., Fo-2	12	1	11		11	7	83	69–90[1]
15% St. Louis C.	12	0			12	11	68	39–90[1]
15% A. B., Strain K_2	12	0			12	12	63	15–90[1]
8% A. B., Strain K_2	4	2	30	28–31	2	2	31	28–34
4% A. B., Strain K_2	5	5	15	8–30	0			
2% A. B., Strain K_2	6	6	11	8–20	0			
1% A. B., Strain K_2	6	6	11	7–17	0			
Dietary supplementation with 0.5 ppm selenium								
8.3% casein	11	0			11	11		90[1]
30% Torula	13	0			13	6		90[1]

[1] Surviving rats were sacrificed after 90 days with the diet.

ralysis" was noted towards the end of the period of lactation in 251 of 403 young rats. Sixty-two of the affected rats died by the 28th day; the rest recovered spontaneously (Goettsch and Pappenheimer, '41). These young were known as vitamin E-low young.

The administration of 10 mg of α-tocopheryl acetate at the beginning of gestation did not protect all rats against muscular dystrophy (Goettsch and Pappenheimer, '41). Seventeen of 177 young rats presented muscular dystrophy towards the end of the lactation period; two of them died by the 28th day and 15 recovered spontaneously.

With the commercial pellet ration, two of 393 young rats developed muscular dystrophy and died before the 28th day, presenting typical lesions. Apparently the pellet ration did not contain sufficient vitamin E to prevent muscular dystrophy in all young lactating rats.

Necrotic liver degeneration. The 8.3% casein diet was fed from the 21st day to 28 young rats of females, reared and bred with the commercial pellet ration and to 56 vitamin E-low young. Ten of the former group died with hepatic necrosis in 16 to 35 days (table 4); the remaining 18 rats were sacrificed at 90 days and presented normal liver and muscle. Fifty-four of the vitamin E-low young died in 5 to 26 days with gross liver lesions; two died after 13 and 50 days with severe muscular dystrophy and presented normal liver. The incidence of hepatic necrosis was high in both groups of rats given the 30% Torula diet, but the time of survival was significantly less in the vitamin E-low young. These results explained why yeast rather than casein diets are used in investigations upon liver necrosis when the initial stores of vitamin E in the rat are not controlled.

The vitamin E-low diets containing 8.3% of casein or 15% of either Torula or Fleischmann yeast induced liver necrosis in a high percentage of vitamin E-low rats (Goettsch, '51). The diet containing 15% of Anheuser Busch yeast, Strain G, protected about half of the rats. At the same concentration the remaining American yeasts (Jos. Schlitz, Strains Fe-2 and

Fo-2; St. Louis Corporation, regular non-debittered dried brewers'; Anheuser Busch, Strain K_2) prevented the disorder in nearly all of the animals. Anheuser Busch yeast, Strain K_2 was fed at lower levels. There was partial protection at the 8% level but none at lower concentrations, under the experimental conditions.

Supplementation of the 8.3% casein and 30% Torula diets with 0.5 ppm of selenium as sodium selenite prevented necrotic changes of the liver in all rats, confirming the observations of Schwarz and Foltz ('57).

Chronic muscular dystrophy in young rats. Muscular dystrophy did not occur by the 90th day in rats that were given the commercial pellet ration until the 21st day (table 4). These rats grew at a more rapid rate than the vitamin E-low young rats given similar diets.

Growth was poor in the vitamin E-low young rats receiving the vitamin E-low protein diets. Early lesions of muscular dystrophy were noted in some of the rats that died with acute dietary necrosis. Many (50 of 58) of the E-low animals that were protected against liver lesions developed muscular dystrophy. Slight paretic symptoms appeared soon after the low-protein diets were given. The symptoms gradually became worse and usually at the time that they were moderately severe, the body weight of the rat remained constant (35 to 65 gm) for several weeks. The rats could not right themselves and, in physical appearance and behavior, resembled adult rats with chronic muscular dystrophy (Einarson and Ringsted, '38). Their disability made it difficult for them to reach food and water. They lost weight gradually and died after a period of inanition. Death occurred in 13 to 89 days after the vitamin E-low, low-protein diets were given. The time of survival was apparently associated with the maximum body weight that the rat was able to attain before the onset of muscular dystrophy. A few rats became emaciated and died without manifesting paretic symptoms. At death there were typical lesions of muscular dystrophy. Surviving rats were sacrificed after 90 days with the diet.

At death the muscle of the rats appeared small in bulk, opaque, brownish and not very contractile; the liver was normal, without fat, except in a few instances; and the gastrointestinal tract was often empty. The central nervous system was not examined. Dr. F. V. Lichtenberg[4] gave the following description of the chronic muscle lesions: "In earlier cases of 'chronic type,' the changes are similar to those observed by Pappenheimer ('39), including myoblastic proliferation and giant cell formation. The animals with the longest survival period (60 to 90 days) show marked atrophy of the striated muscle, fibrous thickening of the connective tissue framework and microscopical scarring. Residual lymphoid and histoid cell infiltration is observed, but decreases markedly in the more chronic specimens. Fatty replacement is not observed at any stage and the similarity to human muscular dystrophy is less apparent than in the rabbit."

The diets containing 0.5 ppm of selenium induced more rapid growth in the rats and a delay in the onset of paretic symptoms. All of the rats with the 8.3% casein diet and one half of those with the 30% Torula diet developed moderately severe symptoms before the 90th day with the diet. When sacrificed they presented normal liver and typical lesions of chronic muscular dystrophy.

SUMMARY

Dietary necrotic liver degeneration developed more readily and more rapidly in young rats with initial low stores of vitamin E, as judged by their spontaneous recovery from acute muscular dystrophy during the period of lactation, than in rats fed a commercial pellet ration until the 21st day. The vitamin E-low necrogenic diets contained 8.3% of crude casein, 15% of Fleischmann yeast or 15 to 30% of Torula food yeast. Certain known strains of American yeasts, at 15% dietary concentration, gave complete or partial protection under the given experimental conditions.

Chronic muscular dystrophy occurred in many of the vitamin E-low young rats that did not die with dietary necrotic liver degeneration. Paretic symptoms appeared

[4] Dr. F. V. Lichtenberg of the Department of Pathology, Peter Bent Brigham Hospital, Boston; formerly of the Department of Pathology, University of Puerto Rico School of Medicine.

during the first few weeks and became progressively worse until death occurred, with normal liver and typical muscular dystrophy. Young rats receiving pellets until the 21st day did not develop the muscle disorder.

Supplementation of the 8.3% casein and 30% Torula diets with 0.5 ppm of selenium as sodium selenite prevented dietary necrotic liver degeneration but failed to protect the young rats against muscular dystrophy.

ACKNOWLEDGMENT

The author is indebted to Dr. F. V. Lichtenberg[5] for microscopic examination of the tissues, pathology summary and criticism of the manuscript.

LITERATURE CITED

Dam, H., G. K. Nielsen, I. Prange and E. Sondergaard 1957 The exudative diathesis produced by Torula Yeast. Experimentia, *13:* 493.

Einarson, L., and A. Ringsted 1938 Effect of chronic vitamin E deficiency on the nervous system and the skeletal musculature in adult rats. A neurotropic factor in wheat germ oil. Levin and Munksgaard, Ejnar Munksgaard, Copenhagen.

Goettsch, M. 1951 The role of vitamin E in the production of nutritional liver injury in rats on low casein diets. J. Nutrition, *44:* 443.

Goettsch, M., and A. M. Pappenheimer 1941 α-Tocopherol requirement of the rat for reproduction in the female and prevention of muscular dystrophy in the young. J. Nutrition, *22:* 463.

Harris, P. L., M. I. Ludwig and K. Schwarz 1958 Ineffectiveness of Factor 3-active selenium compounds in resorption-gestation bioassay for vitamin E. Proc. Soc. Exp. Biol. Med., *97:* 686.

Hove, E. L., G. S. Fry and K. Schwarz 1958 Ineffectiveness of Factor 3-active selenium compounds in muscular dystrophy of rabbits on vitamin E-free diets. Ibid., *98:* 27.

Mattill, H. A. 1947 Antioxidants. Ann. Rev. Biochem., *16:* 177.

Pappenheimer, A. M. 1939 The pathology of nutritional muscular dystrophy in young rats. Am. J. Path., *15:* 179.

Patterson, E. L., R. Milstrey and E. L. R. Stokstad 1957 Effect of selenium in preventing exudative diathesis in chicks. Proc. Soc. Exp. Biol. Med., *95:* 617.

Schultze, M. O. 1960 Nutrition. Ann. Rev. Biochem., *29:* 391.

Schwarz, K. 1951 A hitherto unrecognized factor against necrotic liver degeneration in American Yeast (Factor 3). Proc. Soc. Exp. Biol. Med., *78:* 852.

Schwarz, K., and C. M. Foltz 1957 Selenium as an integral part of Factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc., *79:* 3292.

Stamler, F. W. 1959 Fatal eclamptic disease of pregnant rats fed anti-vitamin E stress diet. Am. J. Path., *35:* 1207.

[5] See footnote 4.

A Mechanism for the Copper-Molybdenum Interrelationship

II. RESPONSE OF LIVER SULFIDE OXIDASE ACTIVITY TO NUTRITIONAL FACTORS[1]

LEWIS M. SIEGEL AND K. J. MONTY
McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland

In the course of study of metabolic alterations in tissues of rats subjected to toxic dietary levels of molybdate, a substantial decrease in the activity of liver sulfide oxidase was noted (Mills et al., '58). This fact became the basis for a postulated explanation of the induction of copper deficiency by toxic levels of molybdate (Halverson et al., '60), wherein the decreased rate of sulfide oxidation would permit, under certain dietary conditions, the abnormal accumulation of sulfide ion. Copper ions consequently would be removed from metabolic availability through the formation of copper sulfide. The extremely low solubility products of the copper sulfides were used to explain the specificity of the nutritional deficiency.

The present studies were undertaken as a partial exploration of the validity of this postulate.

EXPERIMENTAL

Young male albino rats of the Wistar strain[2] were used in all experiments. The basal diet was constituted of casein, glucose, corn oil, salts, and vitamins as described earlier (Halverson et al., '60). This diet contained copper at 1 to 1.5 ppm, as determined by the method of Eden and Green ('40). The same techniques used earlier were invoked to maintain conditions consistent with the feeding of low-copper diets (Halverson et al., '60). Modifications of the basal diet consisted of the addition of one or more of the following materials: 800 ppm of molybdenum as sodium molybdate; 15.6 ppm of copper as cupric acetate; 0.94% of L-cystine; and 0.29% of sulfate as sodium sulfate.

The conventional technique of paired-feeding was modified for the present study. Average food intake of 4 animals of the determinant group was carefully measured daily, and this average determined the amount of food supplied to each member of the paired-fed group for the succeeding 24-hour period.

For the assay of sulfide oxidase, animals were sacrificed by etherization, and their livers perfused rapidly with cold 0.2M sucrose. After excision and weighing, livers were minced, diluted with 12 volumes of cold 0.1M phosphate buffer (pH 7.2), and homogenized for one minute in a Waring Blendor. The homogenate was centrifuged at 12,000 × G for 15 minutes at 0°C, and the supernatant filtered through coarse cheesecloth.

The enzyme reaction was carried out at 37°C for one hour in closed Thunberg tubes, the reaction mixture containing 1 ml of the supernatant from the liver homogenate, 3.5 ml of 0.1M phosphate buffer (pH 7.2), and 0.5 ml of 0.04M sodium sulfide (20 μmoles). Assays for each liver were performed in duplicate. Also carried in duplicate were measurements of "heat stable" activity, using supernatant boiled for three minutes. Assays for thiosulfate endogenous to the liver preparations were invariably negative.

After the incubation, the contents of the reaction vessels were transferred to

Received for publication January 25, 1961.

[1] Contribution no. 321 from the McCollum-Pratt Institute. Supported in part by a contract with the Division of Biology and Medicine, U. S. Atomic Energy Commission.

[2] Obtained from Albino Farms, Red Bank, New Jersey.

centrifuge tubes, and 0.7 ml of 1M cadmium acetate added to terminate further reaction and precipitate unreacted sulfide. A clear supernatant was obtained by centrifugation followed by filtration through Whatman no. 50 paper. One-milliliter aliquots of this filtrate were analyzed for thiosulfate by the colorimetric method of Sörbo ('57). Photometric readings were made at 460 mμ using a Bausch and Lomb Spectronic 20 colorimeter. Protein determinations were carried out on aliquots of the liver preparation using the method of Lowry et al., ('51). Sulfide oxidase activity was expressed as μmoles of thiosulfate formed per milligram of protein per hour. The difference in the activity of nonboiled and boiled preparations was taken to be the sulfide oxidase activity.

RESULTS AND DISCUSSION

Halverson et al., ('60) advanced the hypothesis that the symptoms of copper deficiency observed during molybdate toxicity were due to the formation of copper sulfide in the tissues as a result of an abnormal accumulation of sulfide ion. This postulate was supported by the demonstration that excessive dietary cystine, introduced as early as 7 days after the initiation of feeding of a low-copper diet containing 800 ppm of molybdenum, greatly aggravated the symptoms of anemia and diarrhea. These symptoms could be prevented by raising the level of copper in the diet, or reversed by the injection of copper. The inclusion of 0.29% of sulfate in the diet would alleviate the growth depression due to molybdate as well as correct the conditions of anemia and diarrhea.

The response of liver sulfide oxidase activity to the dietary regimens used by Halverson et al., is presented in figure 1. Exposure to the low-copper, molybdate-containing diet led to a rapid decrease in the level of the enzyme. The difference between molybdate and control animals was maintained for at least 45 days, the longest experimental period used in these investigations. Addition of sulfate to the molybdate diet beginning on the 12th day produced a rapid return of the enzyme to normal levels. In contrast, the addition of cystine to the molybdate diet did not lead to a restoration of enzyme activity. The response of liver sulfide oxidase to these dietary parameters correlates well with the changes in the concentration of circulating hemoglobin observed by Halverson et al. ('60).

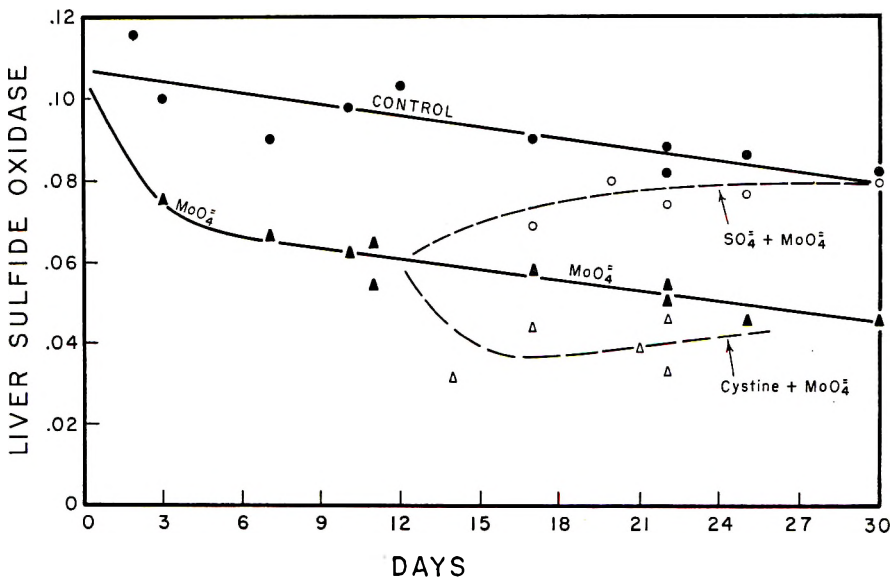

Fig. 1 Influence of dietary molybdenum, cystine and sulfate on the sulfide oxidase activity of rat liver preparations; composition of diets described in text; supplementations of the molybdate diet with cystine or sulfate was begun on the 12th day; sulfide oxidase activity expressed as μmoles of thiosulfate formed/mg protein/hour.

The 4th parameter to be considered was the dietary level of copper. In table 1 is presented a summary of the response of liver sulfide oxidase activity to molybdate, cystine and sulfate when added to diets low in copper (1.0 to 1.5 ppm) or high in copper (16.5 to 17.0 ppm). Several effects are apparent. First, the addition of 15.6 ppm of copper to each of the low-copper diets led to a higher activity of sulfide oxidase. Application of the analysis of variance to each of the proper diet pairs (with and without copper supplementation) indicated a high level of confidence for these differences ($P < 0.001$). Also, the addition of cystine to a high-copper basal diet resulted in a substantial increase in sulfide oxidase activity. A similar effect was noted when molybdate was present in the high-copper diet. In the absence of copper supplementation, however, cystine produced a decrease in sulfide oxidase when added to the high-molybdate diet. The effect of the addition of cystine to a low-copper basal diet is not indicated in table 1. A separate experiment demonstrated that this, too, leads to a decrease in activity of the enzyme (basal, 0.091 ± 0.006; basal + cystine, 0.079 ± 0.007).

Animals supplied with the high-molybdate diets, characteristically demonstrated a substantially reduced food intake, and consequently a retarded rate of weight gain or even a loss of weight. The drop in sulfide oxidase activity is related more to the reduced food intake than to the ingestion of molybdate (table 2). When the low-copper diet was supplied, the change in enzyme activity could be duplicated with paired-fed animals which received no molybdate; with the high-copper diets, however, the magnitude of the molybdate effect could not be duplicated by paired-feeding. In both experiments, the body weights of the paired-fed animals closely paralleled those of the animals receiving the toxic level of molybdate.

The experiments reported here suggest several relationships between the activity of liver sulfide oxidase and the nutritional status of the animal. First, food restriction promotes a substantial decrease in the

TABLE 1

Influence of dietary copper, molybdenum, cystine and sulfate on the sulfide oxidase activity[1] of rat liver preparations

Dietary supplement	Low-copper[2]	High-copper[3]
—	0.085 ± 0.003[4](6)[5]	$0.104 \pm 0.005(5)$
Cystine		$0.156 \pm 0.012(5)$
Sulfate		$0.105 \pm 0.008(5)$
Molybdate	$0.050 \pm 0.004(7)$	$0.064 \pm 0.003(5)$
Molybdate + cystine	$0.044 \pm 0.006(5)$	$0.087 \pm 0.007(5)$
Molybdate + sulfate	$0.080 \pm 0.008(6)$	$0.109 \pm 0.010(5)$

[1] Sulfide oxidase activity is expressed as μmoles of thiosulfate formed/mg protein/hour.
[2] Time of dietary additions same as in figure 1. Animals sacrificed randomly during days 20 to 45.
[3] Additions of cystine and sulfate on 24th day. Animals sacrificed randomly during days 38 to 45.
[4] Standard deviation of the mean $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.
[5] Number of animals used is indicated in parentheses.

TABLE 2

Influence of restricted food intake on sulfide oxidase activity[1] of rat liver preparations[2]

Dietary regimen	Low-copper	High-copper
Basal, ad libitum	0.093 ± 0.006[3](4)[4]	$0.109 \pm 0.011(4)$
Basal + molybdenum, ad libitum	$0.067 \pm 0.005(4)$	$0.065 \pm 0.006(4)$
Basal, paired-fed to molybdenum animals	$0.066 \pm 0.006(5)$	$0.076 \pm 0.006(4)$

[1] Micromoles thiosulfate formed/mg protein/hour.
[2] Experimental design described in text.
[3] Standard deviation of the mean.
[4] Number of animals used is indicated in parentheses.

level of the enzyme activity. It is not possible with the present data to establish whether this decrease is due to a change in apoenzyme concentration or to an altered availability of one or more cofactors. Mills et al. ('58), were unable to explain the decrease in sulfide oxidase activity during molybdenum toxicity in terms of a lack of hypoxanthine, an indicated cofactor for the enzyme (Ichihara, '59).

A second generalization arising from these experiments is that the level of sulfide oxidase activity is consistently higher when diets high in copper are used. This effect is independent of the dietary levels of molybdate, cystine or sulfate. The correlation of enzymatic activity with dietary supply of copper suggests that the enzyme may be dependent in some way upon copper *in vivo*. In this respect, the inhibition of liver sulfide oxidase by cyanide is prevented more satisfactorily by copper than by any other cation examined (Ichihara, '59). Efforts are currently under way in this laboratory to elucidate this relationship between sulfide oxidase and copper.

Also the addition of cystine to high-copper diets (with or without molybdate) has been observed to lead to a rise in sulfide oxidase activity. Since one of the pathways for the metabolism of cystine leads to the formation of sulfide, it is possible that the level of sulfide oxidase activity is modified by the availability of its substrate, a theory that demands further experimental exploration.

The present findings have implications for the understanding of the copper-molybdenum interrelationship in rats. The results in general are such as would be necessary to support the hypothesis that this interrelationship is due to a loss of available copper as the sulfide salt, resulting from inadequate rates of sulfide oxidation in the tissues (Halverson et al., '60). The combinations of dietary stimuli that induce the symptoms of copper deficiency also produce the most profound decreases in liver sulfide oxidase activity. The additional drop in enzyme activity upon the addition of cystine to a molybdate-containing diet may be construed as a cascading situation wherein the decrease of an enzyme dependent upon copper leads to the induction of a copper shortage in the tissues. This lack of copper availability in turn leads to a further decrease in the level of the enzyme. In this situation cystine would serve as a precursor of sulfide.

Since the drop in enzyme activity may be reproduced in paired-fed animals supplied with low-copper diets, the postulate under discussion would predict that anemia would result from suppling these paired-fed animals with dietary cystine. A preliminary experiment does not support this prediction.[3] Thus, the postulate of Halverson et al. ('60) may be useful to the understanding of the copper-molybdenum interrelationship, but is probably not an adequate explanation.

SUMMARY

The *in vivo* response of liver sulfide oxidase to a number of nutritional stimuli is reported. The decreased level of the enzyme accompanying molybdate toxicity was attributable largely to the decreased food intake of the animals. Evidence was presented to indicate a dependence of the enzyme upon copper and an adjustment of enzyme activity to substrate level. The results were discussed in terms of a postulated role for the enzyme in the induction of copper deficiency by toxic levels of molybdate.

LITERATURE CITED

Eden, A., and H. H. Green 1940 Micro-determination of copper in biological material. Biochem. J., *34:* 1202.

Halverson, A. W., J. H. Phifer and K. J. Monty 1960 A mechanism for the copper-molybdenum interrelationship. J. Nutrition, *71:* 95.

Ichihara, A. 1959 Oxidation of sulfide in beef liver. Memoirs Inst. Protein Research, Osaka Univ., *1:* 177.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.

Mills, C. F., K. J. Monty, A. Ichihara and P. B. Pearson 1958 Metabolic effects of molybdenum toxicity in the rat. J. Nutrition, *65:* 129.

Sörbo, B. 1957 A colorimetric method for the determination of thiosulfate. Biochim. Biophys. Acta, *23:* 412.

[3] Unpublished data.

Influence of Diet on Serum Cholesterol in the Chick[1,2]

J. E. MARION, H. M. EDWARDS, JR. AND J. C. DRIGGERS
Poultry Department, University of Georgia, Athens, Georgia

Many investigations have been carried out with chickens in an attempt to relate various dietary factors to cholesterolemia. Increasing the intake of dietary protein has been shown to be more effective than decreasing fat intake in reducing serum cholesterol of male chickens (Kokatnur et al., '58). Other workers have observed an inverse relationship between dietary protein level and serum cholesterol in growing chicks (Nishida et al., '58; Johnson et al., '58; March and Biely, '59; Fisher et al., '59; Nishida et al., '60). Generally, this relationship exists only when the protein level ranged from adequate to suboptimal.

The elevation of serum cholesterol by increasing dietary cholesterol may be partially dependent on the protein and fat content of the diet. Peterson et al. ('53) obtained a more marked increase in serum cholesterol due to dietary cholesterol when a vegetable fat was added to the diet. Nutrient imbalance due to the combination of low protein, appreciable quantities of fat, and dietary cholesterol has been effective in raising the serum cholesterol level of chicks (Nishida et al., '58; March and Biely, '59; Fisher et al., '59).

It appears that little consideration has been given to the influence of dietary calorie content on serum cholesterol as affected by protein, fat and cholesterol. The following experiments were conducted to determine the effect of energy and some other nutrients in the diet on serum cholesterol in growing chicks.

EXPERIMENTAL

Day-old White Plymouth Rock cockerel chicks were obtained from a commercial source, individually wing-banded and grown to 4 weeks of age in metal battery brooders. Three groups of 8 chicks each were placed in randomized pens and maintained with each experimental diet. Feed and water were supplied ad libitum.

The composition of a 25% protein control diet containing 3.25 Cal./gm of metabolizable energy follows. This low-fat diet contains the following ingredients expressed as grams per 100 gm: glucose monohydrate, 66.09; isolated soybean protein,[3] 26.67; D,L-methionine, 0.67; glycine, 0.33; cellulose,[4] 1.00; $Ca_3(PO_4)_2$, 3.00; $CaCO_3$, 0.09; NaCl, 0.75; KCl, 0.60; $MgSO_4$, 0.22; choline chloride (70%), 0.29; plus the following expressed as milligrams per 100 gm: vitamin A (384,000 USP units/gm), 2.04; vitamin E (134,400 I.U./pound), 22.3; vitamin D_3 (950,000 USP units/gm), 0.06; thiamine·HCl, 1.98; riboflavin, 1.98; Ca pantothenate, 3.30; pyridoxine·HCl, 0.99; niacin, 3.96; folic acid, 0.66; biotin, 0.07; vitamin B_{12} (1 mg/gm), 3.30; *p*-aminobenzoic acid, 16.5; inositol, 165.0; menadione (63%), 0.53; $FeSO_4 \cdot 7H_2O$, 11.0; $CuSO_4 \cdot 5H_2O$, 1.1; $CoCl_2 \cdot 6H_2O$, 1.1; KI, 1.1; $Na_2MoO_4 \cdot 2H_2O$, 0.11; $ZnSO_4 \cdot 7H_2O$, 16.5; $MnSO_4 \cdot H_2O$, 17.0; Santoquin,[5] 15.0. Substitutions were made in this basal diet by varying isolated soybean protein, glucose, corn oil, cellulose and cholesterol. The dietary treaments

Received for publication January 27, 1961.

[1] Journal Paper no. 149 of the College Experiment Station, University of Georgia, College of Agriculture Experiment Stations.

[2] This work was supported in part by research grant no. 3766 from the National Institutes of Health, U. S. Public Health Service, Department of Health, Education, and Welfare.

[3] Archer-Daniels-Midland Company, Cincinnati, Ohio.

[4] Solka Floc, Brown Company, Berlin, New Hampshire.

[5] Trade name for 1,2 dihydro-6 ethoxy-2,2,4-trimethyl quinoline, Monsanto Chemical Company, St. Louis.

TABLE 1

Effect of protein and fat content of diet on serum cholesterol

Diet no.	Dietary treatment: Corn oil	Protein	Calories	C:P	4 Week weight	Serum cholesterol
	%	%	*Cal./gm*		*gm*	*mg/100 ml*
1	0	18.4	3.16	0.172	389	118
2	0	21.2	3.17	0.150	416	116
3	0	25.0	3.18	0.127	407	124
4	0	29.0	3.19	0.110	407	137
5	0	33.0	3.20	0.097	383	117
6	10	25.0	3.73	0.149	480	153
7	20	25.0	4.29	0.172	458	145
8	10	29.0	3.74	0.129	459	152
9	20	29.0	4.30	0.148	462	159

Grouping of treatment results[1]

Diet no.	2	5	1	3	4	7	8	6	9
Serum cholesterol	116	117	118	124	137	145	152	153	159
Diet no.	5	1	3	4	2	7	8	9	6
Body weights	383	389	407	407	416	458	459	462	480

[1] Two values not underscored by the same line are significantly different ($P < 0.05$).

TABLE 2

Effect of protein, energy and fat content of diet on serum cholesterol

Diet no.	Dietary treatment: Corn oil	Protein	Calories	4 Week weight	Serum cholesterol[1]
	%	%	*Cal./gm*	*gm*	*mg/100 ml*
1	0	15	2.81	379	127
2	10	15	2.81	401	133
3	0	15	3.25	331	128
4	10	15	3.25	433	149
5	0	25	3.25	480	128
6	10	25	3.25	518	133
7	10	25	3.80	539	155

Grouping of treatment results[2]

Diet no.	3	1	2	4	5	6	7
Body weights	331	379	401	433	480	518	539

[1] Treatment differences not significant ($P < 0.05$).

[2] Two values not underscored by the same line are significantly different ($P < 0.05$).

for each experiment are presented with the experimental results in tables 1, 2 and 3.

Individual body weights were obtained at two and 4 weeks, feed consumption was measured and feed efficiency calculated. At 4 weeks of age, a 5-ml sample of blood was obtained by heart puncture from 12 chicks receiving each diet. The serum was separated by centrifugation, extracted with an acetone ethanol solution (1:1 by volume) and this extract analyzed for total cholesterol by the method of Schoenheimer and Sperry ('34). All serum cholesterol values and growth data were subjected to a statistical analysis and in experiments 1 and 2, treatment significance was determined by the multiple range test of Duncan ('55).

TABLE 3

Effect of protein, energy, fat and cholesterol content of the diet on serum cholesterol

Dietary treatment				4 Week weight	Serum cholesterol
Protein	Calories	Corn oil	Cholesterol		
%	*Cal./gm*	%	%	*gm*	*mg/100 ml*
15	2.81	0	0	325	122
15	2.81	0	1	336	121
15	2.81	10	0	433	127
15	2.81	10	1	418	435
15	3.25	0	0	308	148
15	3.25	0	1	316	124
15	3.25	10	0	428	126
15	3.25	10	1	442	561
15	3.69	10	0	404	163
15	3.69	10	1	408	531
25	2.81	0	0	408	124
25	2.81	0	1	406	134
25	2.81	10	0	416	119
25	2.81	10	1	419	242
25	3.25	0	0	469	130
25	3.25	0	1	469	131
25	3.25	10	0	513	119
25	3.25	10	1	526	334
25	3.69	10	0	558	137
25	3.69	10	1	544	280

RESULTS AND DISCUSSION

The results of a trial designed to determine the effect of varying the protein level on serum cholesterol in the presence and absence of corn oil are shown in table 1. Five protein levels were used with the low-fat diets and two with the fat-supplemented diets. Serum cholesterol was not affected by varying the protein in either the low-fat or fat-supplemented diets, but was increased in all instances where corn oil was added. It would appear from the results that the protein level of the diet had no effect on the serum cholesterol level either in the presence or absence of corn oil. Regardless of protein level, a statistically significant increase in growth rate resulted from the addition of corn oil in this trial.

The increase in serum cholesterol obtained in trial one from the addition of corn oil could have been caused by the increase in energy content of these diets, since no effort was made to keep the diets isocaloric. Therefore, a trial was designed to make additions of corn oil isocalorically at two protein levels using two energy levels at each protein level. The results of this trial are presented in table 2. Serum cholesterol values were not significantly affected by the dietary treatments but the following changes were noted. Levels of serum cholesterol did not appear to be influenced by the isocaloric addition of corn oil to the low-protein, low-energy diet (2.81 Cal. per gm), but were elevated by adding corn oil to the median energy diet (3.25 Cal. per gm) at the same protein level. At the higher protein level an increase in serum cholesterol from the addition of corn oil was obtained only with the high-energy diet (3.80 Cal. per gm). The average weight gains were higher when chicks received diets supplemented with corn oil, with the greatest response in weight being made when they received the diets with the isocaloric addition of corn oil to the low-protein median-energy diet.

An attempt to clarify further some of the interrelationships between protein, energy and fat was made by conducting a $2 \times 3 \times 2 \times 2$ factorially designed experiment with variables of protein, energy, fat and cholesterol content of the diet, respectively. All combinations of these variables were used except 4 high-energy, low-fat diets which were impossible to formulate with the ingredients used.

The results of this trial are shown in table 3 and a statistical analysis of the data is presented in table 4. The effect

TABLE 4

Statistical analysis of results obtained in trial 3

Source of variation	Body weight		Serum cholesterol	
	d. f.	Mean square	d. f.	Mean square
P (protein)	1	1,003,671[1]	1	197,258[1]
E (energy)	2	90,539[1]	2	39,200[1]
PE	2	165,143[1]	2	5,066
F (fat)	1	312,118[1]	1	251,948[1]
PF	1	127,439[1]	1	143,611[1]
EF	1	29,357[1]	1	20,073
PEF	1	13,044	1	65
C (cholesterol)	1	194	1	613,048[1]
CP	1	989	1	107,705[1]
CE	2	1,366	2	24,713
CPE	2	713	2	317
CF	1	23	1	252,107[1]
CPE	1	2,070	1	135,527[1]
CEF	1	1,538	1	48,594[1]
Error	385	4,094	212	9,131
Total	405		232	
R^2	0.56		0.70	

[1] Denotes significance ($P < 0.01$).

of the dietary variables and certain interactions on body weight and serum cholesterol was determined. Serum cholesterol levels did not appear to be influenced by dietary protein, cholesterol or energy content in the absence of fat. The low-protein (3.25 Cal. per gm) diet without cholesterol or fat had an unusually high cholesterol value but this appears to have been an anomaly that cannot be related to dietary manipulation. The addition of corn oil at each protein level resulted in an increase in serum cholesterol only when dietary cholesterol was present or the energy content of the diet was very high. When both dietary cholesterol and corn oil were present, serum cholesterol levels were high, but did not appear to be influenced appreciably by the level of energy. An increase in dietary protein, however, lowered the serum cholesterol in the presence of fat. This decrease was marked when both fat and cholesterol were present in the diet. The relationship observed between the levels of dietary protein and serum choelsterol by various workers is, therefore, believed to exist only when some source of dietary fat is present. The mechanism whereby dietary protein lowers serum cholesterol remains obscure as no apparent relationship was noted between actual protein consumption and serum cholesterol values. This observation differs from that of Kokatnur et al. ('58a) who related cholesterol elevation in the sera of mature birds to a reduction in total protein consumption.

The results obtained indicate that in the absence of dietary fat, serum cholesterol cannot be markedly altered by variations of protein, energy and cholesterol content of the diet. This suggests that the effect of energy to protein ratio on serum cholesterol observed by Kokatnur et al. ('58) does not exist in diets that are very low in fat.

The isocaloric addition of corn oil at both protein levels resulted in an increase in body weight gains, with the greatest increase occurring on the low-protein diet at both the low and median energy levels. It appears that the chicks fed the low-fat diets had a fatty acid deficiency such as that reported by Machlin and Gordon ('60) who were able to produce this deficiency as early as one week of age. The data indicate that the protein level of the diet influenced the severity of this deficiency as a greater response in weight gain was produced by the isocaloric addition of corn oil at the low-protein level.

SUMMARY

Variations in dietary protein, energy and cholesterol failed to influence serum cholesterol levels in growing chicks when

fed a diet very low in fat. Increases of serum cholesterol were obtained by the addition of corn oil to a diet containing cholesterol.

An inverse relationship between dietary protein and serum cholesterol was noted only when corn oil was present in the diet. This relationship was pronounced when cholesterol was incorporated in a corn oil supplemented diet. Growth responses were observed when corn oil was added isocalorically to the low-fat diet indicating that the chicks fed the low-fat diet may have developed a fatty acid deficiency. Greater growth response to the addition of corn oil occurred with diets low in protein which suggests that the protein level of the diet may greatly influence fatty acid deficiency.

LITERATURE CITED

Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.

Fisher, H., A. Feigenbaum, G. A. Leveille, H. S. Weiss and P. Griminger 1959 Biochemical observations on aortas of chickens. Effect of different fats and varying levels of protein, fat and cholesterol. J. Nutrition, 69: 163.

Johnson, D., Jr., G. A. Leveille and H. Fisher 1958 Influence of amino acid deficiencies and protein level on the plasma cholesterol of the chick. Ibid., *66:* 367.

Kokatnur, M., N. T. Rand and F. A. Kummerow 1958 Effect of energy to protein ratio on serum and carcass cholesterol levels in chicks. Circulation Res., 6: 424.

Kokatnur, M., N. T. Rand, F. A. Kummerow and H. M. Scott 1958a Effect of dietary protein and fat on changes of serum cholesterol in mature birds. J. Nutrition, 64: 177.

Machlin, L. J., and R. S. Gordon 1960 The requirement of the chicken for certain unsaturated fatty acids. Poultry Sci., 39: 1271.

March, B. E., and J. Biely 1959 Dietary modification of serum cholesterol in the chick. J. Nutrition, 69: 105.

Nishida, T., A. Ueno and F. A. Kummerow 1960 Effect of dietary protein on the metabolism of sodium acetate-1-C^{14} in chicks. Ibid., *71:* 379.

Nishida, T., F. Takenaka and F. A. Kummerow 1958 Effect of dietary protein and heated fat on serum cholesterol and beta-lipoprotein levels, and on the incidence of experimental atherosclerosis in chicks. Circulation Res., 6: 194.

Peterson, D. W., E. A. Shneour, N. F. Peek and H. W. Gaffey 1953 Dietary constituents affecting plasma and liver cholesterol in cholesterol-fed chicks. J. Nutrition, *50:* 191.

Schoenheimer, R., and W. M. Sperry 1934 Micromethod for determination of free and combined cholesterol. J. Biol. Chem., *106:* 745.

Nitrogen Intake and Ribonuclease Activity in Normal and Tumor-Bearing Rats[1]

J. B. ALLISON, R. W. WANNEMACHER, JR., L. P. PARMER AND R. GOMEZ
Bureau of Biological Research, Rutgers University, New Brunswick, New Jersey

Previous studies have demonstrated that the development of protein in the liver and tumors of rats was maximal when the ribonuclease activity of the tissue was minimal (Zigman and Allison, '59). These and other data have suggested a correlation between ribonuclease activity, ribonucleic acid (RNA) and the synthesis of normal and tumor tissue proteins (Hoagland et al., '58; Clark et al., '57). The following studies were designed to determine in more detail the effect of dietary protein intake upon the growth of the Walker 256 carcinosarcoma, ribonuclease activity, ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and protein content of tumor and liver tissues. The previous data demonstrated lipemia was associated with the growth of the tumor in the tumor-bearing animal or with the depletion in protein reserves in the normal animal (Allison and Wannemacher, '59). Further data have been obtained, therefore, to correlate nitrogen intake with serum cholesterol, phospholipid, and lipoproteins and glycoproteins in normal and tumor-bearing rats.

METHODS

Male Wistar rats weighing 100 to 150 gm were fed a diet containing zero, 9, 12, 18, 25, 35 or 50% of casein (Allison et al., '54). In each diet group, 10 rats received transplants of Walker 256 carcinosarcoma and 10 animals were controls. Fifteen days after transplantation, the tumor-bearing and control rats were sacrificed and samples of liver, tumor and serum were taken for chemical analysis. Food intake and the body weight of each animal were recorded throughout the experiment.

Serum proteins were separated by paper electrophoresis and stained with alcoholic bromophenol blue. Lipid was stained by oil red O (Jencks et al., '55). The glycoproteins were analyzed by the technic of Kőiw and Grőnwall ('52). Total serum proteins were determined by the Biuret method of Layne ('57), and ribonuclease activity was estimated by the technic of Brody ('57).

The liver and tumor tissues from each rat were homogenized into a 20% suspension with 0.1M phosphate buffer, pH 7.4. This suspension was used to determine total protein by the microKjeldahl assay of Pregl and Roth ('35), ribonuclease activity by the spectrophotometric method of Brody ('57), and nucleic acid phosphorus concentration by the procedure of Schmidt and Thannhauser ('45).

RESULTS

The data in figure 1 illustrate the increase in RNA phosphorus and total protein in the liver of rats that were fed increasing intakes of casein nitrogen over a period of 15 days. A decrease of ribonuclease activity in the liver and serum was associated with this rise in RNA. Liver DNA-phosphorus per weight of tissue was independent of nitrogen intake. These data support the concept that the development of the protein reserves is correlated with the quantity of RNA and that this quantity may be correlated with the ribonuclease activity. Reduced protein synthesis then could be associated with a high activity of this enzyme system. The data in figure 2 demonstrate

Received for publication January 30, 1961.
[1] The studies were supported by a grant from the American Cancer Society.

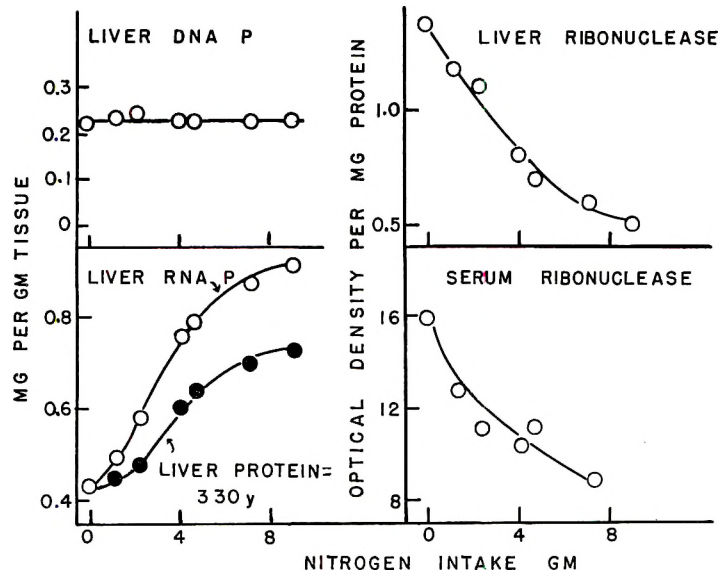

Fig. 1 The effect of casein nitrogen intake over a period of 15 days upon RNA-phosphorus, DNA-phosphorus and protein in the liver and upon ribonuclease activity in the liver and serum of normal rats.

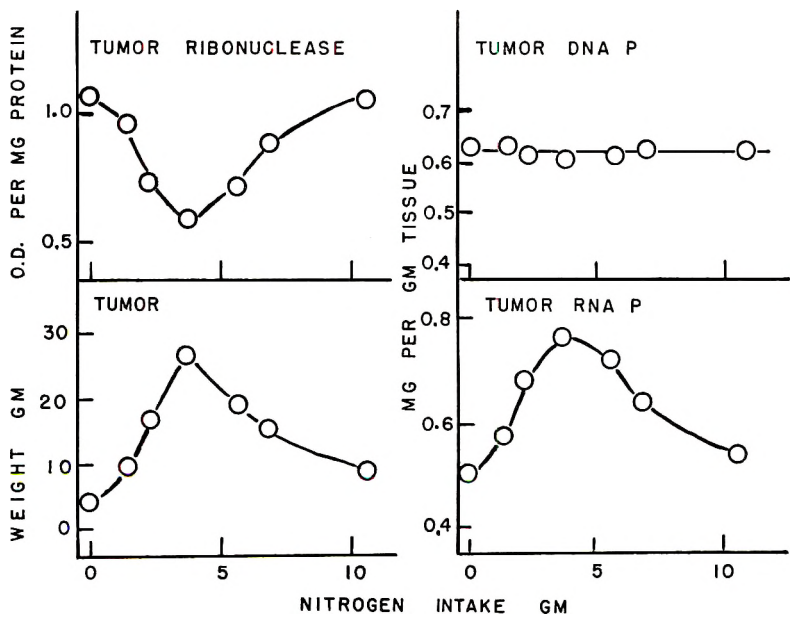

Fig. 2 The effect of casein nitrogen intake over a period of 15 days upon the growth of Walker 256 carcinosarcoma and the RNA-phosphorus, DNA-phosphorus and the ribonuclease activity of the tumor.

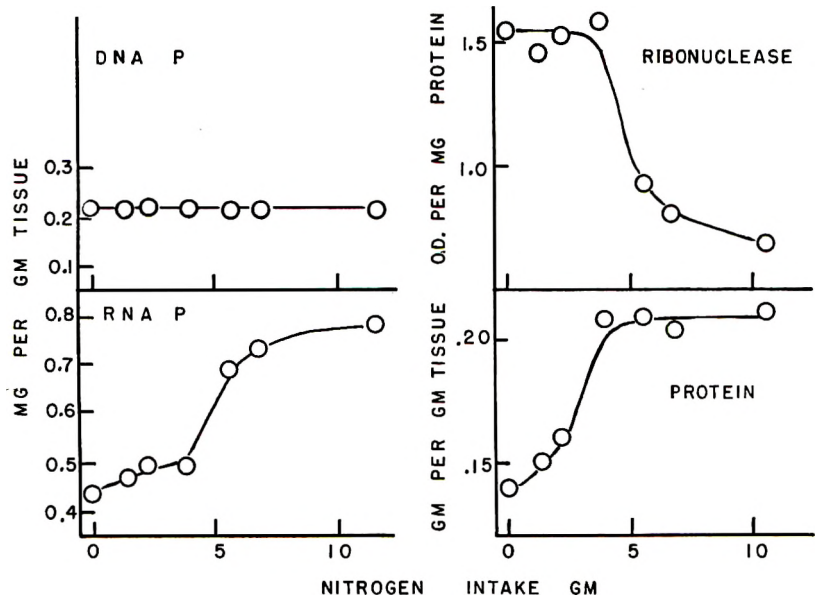

Fig. 3 The effect of casein nitrogen intake over a period of 15 days upon RNA-phosphorus, DNA-phosphorus, ribonuclease activity and protein in the liver of tumor-bearing rats.

that there was an optimum casein nitrogen intake for tumor growth (Allison and Fitzpatrick, '60). Similarly, there was maximum tumor RNA phosphorus and minimum ribonuclease activity at the point of optimal growth of the tumor but the milligrams of DNA-phosphorus per gram of tumor tissue was not altered by the casein nitrogen intake. The DNA-phosphorus in the tumor tissue was approximately three times that observed in liver tissue, this high DNA content of the tumor being correlated with the rapid growth of the neoplasm. These results involving DNA are similar to those presented by Schneider and Klug ('46).

Data in figure 3 illustrate the DNA-phosphorus, RNA-phosphorus, ribonuclease activity and protein in the liver of tumor-bearing rats at various nitrogen intakes. These data may be compared with those in figure 1 for normal controls. Data in figure 3 demonstrate that at low nitrogen intake the ribonuclease activity was higher and the RNA was lower in the liver of tumor-bearing animals than in the normal controls. As the nitrogen intake was increased beyond the optimal for tumor growth there was a marked drop in ribonuclease activity and a rise in RNA, which was correlated with a rise in liver protein. These observations support previous reports that increased nitrogen intake protected the body from the depleting effects of the tumor (Allison, '56).

Data in table 1 record the effect of casein nitrogen intake upon serum proteins and the lipid and carbohydrate migrating electrophoretically with these proteins in normal and tumor-bearing rats. The depleting effect of the tumor at low nitrogen intakes is illustrated by the reduced serum albumin in the tumor-bearing animals when compared with the controls. At higher nitrogen intakes the serum albumin was not altered by the tumor. These observations demonstrate an increase in the lipid migrating with the α-globulin in normal rats fed a protein-free diet as compared with those fed 4 or more grams of nitrogen ($P < 0.01$). The rise in lipid migrating electrophoretically with the globulins in tumor-bearing rats reached a maximum at the point of optimal growth of the tumor. This rise is particularly marked in the β-globulin fraction ($P < 0.01$). Similarly, the carbohydrate associated with serum protein

TABLE 1

Effect of casein nitrogen intake upon serum proteins, and the lipid and carbohydrate migrating electrophoretically with those proteins in normal and tumor-bearing rats

Nitrogen intake	Serum proteins					Lipid			Glyco				
	Albumin	Alpha$_1$	Alpha$_2$	Beta	Gamma	Alpha$_1$	Alpha$_2$	Beta	Albumin	Alpha$_1$	Alpha$_2$	Beta	Gamma
gm	*gm/100 ml*					*mg/100 ml*			*mg/100 ml*				
Normal													
0	1.31	0.94	0.74	0.98	0.49	303	95	217	6	28	15	12	4
1.3	2.02	0.95	0.67	0.76	0.55	280	47	232	7	29	24	20	5
2.3	2.20	0.95	0.59	0.86	0.50	187	97	146	5	28	16	12	5
4.1	2.66	1.07	0.66	1.02	0.52	91	124	183	8	30	17	15	5
4.8	2.81	0.79	0.47	0.91	0.68	126	118	157	7	35	20	15	6
7.3	2.65	1.08	0.58	0.86	0.57	147	94	139	10	52	23	10	5
9.1	2.53	0.99	0.50	0.91	0.61	152	64	179	9	40	18	16	7
Tumor-bearing													
0	0.89	0.62	0.49	0.46	0.49	333	109	349	6	32	13	15	8
1.4	1.80	0.81	0.48	0.87	0.35	222	100	266	12	48	23	25	7
2.2	2.29	0.88	0.58	1.03	0.34	210	180	1034	10	47	23	20	14
3.9	2.31	0.89	0.71	0.79	0.47	742	745	2349	12	64	33	57	21
5.6	2.28	0.92	0.64	0.86	0.38	306	257	295	13	64	23	28	8
6.8	2.43	0.98	0.56	0.95	0.80	267	275	165	7	42	15	18	7
10.6	2.52	0.93	0.50	0.83	0.61	99	150	127	5	36	11	14	5

fractions tended to become maximum at optimal growth of the tumor. Possibly this lipemia is associated, at least in part, with the stress of tumor growth and depletion in proteins which results in the alteration of oxidation and transport mechanisms (Allison and Wannemacher, '60).

SUMMARY

1. The RNA-phosphorus and protein content of the liver increased and the activity of ribonuclease decreased as casein nitrogen intake of normal rats was increased. The DNA-phosphorus content of the liver was independent of nitrogen intake.

2. There was an optimum intake of casein nitrogen for maximal growth of the Walker 256 carcinosarcoma transplanted into rats. The RNA-phosphorus was maximal and the ribonuclease activity of the tumor was minimal at the point of maximal growth of the tumor. The DNA-phosphorus was high in the tumor tissue and independent of the nitrogen intake.

3. The growth of the tumor at low-nitrogen intake resulted in a reduced RNA-phosphorus and an increased ribonuclease activity of the liver when compared with normal controls. The tumor had no effect upon DNA-phosphorus of the liver.

4. Lipemia associated with the growth of the tumor was maximal during maximal growth of the tumor.

LITERATURE CITED

Allison, J. B. 1956 Optimal nutrition correlated with nitrogen retention. Am. J. Clin. Nutrition, *4:* 662.

Allison, J. B., and R. W. Wannemacher, Jr. 1959 Plasma proteins and plasma lipid. In: Proteides of Biological Fluids (Proc. 7th Colloquim, Bruges, 1959). Elsevier Publishing Co., Amsterdam, p. 284.

——— 1960 Lipemia associated with protein depletion. Conference on Protein Needs, Committee on Protein Malnutrition, National Research Council, National Academy of Sciences —National Research Council, Washington, D. C., in press.

Allison, J. B., R. W. Wannemacher, Jr., R. Hilf, J. F. Migliarese and M. L. Crossley 1954 Dietary protein and tumor-host relationship in the rat. J. Nutrition, *54:* 593.

Allison, J. B., and W. H. Fitzpatrick 1960 Dietary Proteins in Health and Disease. Charles C Thomas, Springfield, Illinois, p. 36.

Brody, S. 1957 Ribonuclease activity and cell growth. Biochem. biophys. acta, *24:* 502.

Clark, C. M., J. D. Naismith and H. N. Munro 1957 Influence of dietary protein on the incorporation of labeled glycine and phosphorus into the ribonucleic acid of rat liver. Ibid., *23:* 587.

Hoagland, M. B., M. L. Stephenson, J. F. Scott, L. I. Hecht and P. C. Zamecnik 1958 A soluble intermediate in protein synthesis. J. Biol. Chem., *231:* 241.

Jencks, W. P., E. L. Durrum and M. P. Jetton 1955 Paper electrophoresis as a quantitative method: The staining of serum lipoproteins. J. Clin. Invest., *34:* 1437.

Kŏiw, E., and A. Grŏnwall 1952 Staining of protein bound carbohydrate after electrophoresis on filter paper. Scandinav. J. Clin. Lab. Invest., *4:* 244.

Layne, E. 1957 Spectrophotometric and turbidimetric methods for measuring proteins. III. Biuret method. In: Methods in Enzymology, vol. 3. Academic Press, Inc., New York, p. 450.

Pregl, F., and H. Roth 1935 Die quantitative organische Mikroanalyse. J. Springe, Verlage, Berlin, p. 84.

Schmidt, G., and S. J. Thannhauser 1945 A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem., *161:* 83.

Schneider, W. C., and H. L. Klug 1946 Phosphorus compounds in animal tissues. IV. The distribution of nucleic acids and other phosphorus-containing compounds in normal and malignant tissue. Cancer Res., 6: 691.

Zigman, S., and J. B. Allison 1959 Ribonuclease activity of protein-depleted and tumor-bearing rats. Ibid., *19:* 1105.

Vitamin K in the Nutrition of Mink[1,2]

HUGH F. TRAVIS,[3,4] ROBERT K. RINGER AND PHILIP J. SCHAIBLE
Fur Animal Project, Department of Poultry Science, Michigan State University, East Lansing, Michigan

Most mammals obtain part of their vitamin K requirements from synthesis by their own intestinal bacterial flora. However, the anatomical and physiological limitations of the mink (Mustela vison) leave doubt as to its ability to obtain sufficient vitamin K in this manner. The digestive tract is only 4 times the body length, there is no caecum, and the relatively undifferentiated large intestine is only about one-third of the body length (Kainer, '54). Food passage is rapid, averaging less than two hours in mink with empty digestive tracts, and slightly more than two hours when food is continuously available (Wood, '56). Thus, the conditions for bacterial synthesis in the gut would appear more limited in mink than in most mammals.

It has also been established that some mink containing the Aleutian genes for color (aa) have an unexplained bleeding from the mouth and digestive tract (Helmboldt and Jungherr, '58) which could be associated with a deranged blood clotting mechanism.

This study was, therefore, initiated for the following reasons: (1) to determine if mink require a dietary source of vitamin K; (2) to observe what effects certain antibiotics and medicaments would have upon blood clotting time; and (3) to compare the whole blood prothrombin time of dark mink to that of mink containing the Aleutian genes (aa) for color.

GENERAL PROCEDURES

Animals were supplied with the experimental ration for a period sufficient to deplete their body stores of vitamin K, and then the effects of vitamin K in the ration were evaluated by observing the increase in prothrombin times.

The method of determination was the whole blood prothrombin time as modified by Frost et al. ('56) using lyophilized extracts as the source of thromboplastin.[5] To obtain more uniform results, animals were bled by heart puncture using siliconized needles, tubes and syringes.

Mink were housed individually in conventional outdoor pens of 1 × 1-inch wire mesh with aluminum feeders and waterers. Chicks used in the biological assay of the basal mink ration for vitamin K were raised in electrically-heated batteries with a wire floor mesh of ⅓ × ⅓-inch. In order to minimize coprophagy and/or bacterial synthesis, the pens, feeders and waterers of the mink and chicks were cleaned daily and the purified rations were kept refrigerated until fed. Data were subjected to analyses of variance, multiple range and multiple F tests.

Experiment 1. Thirty female sapphire (homozygous for the Aleutian gene) and 30 female dark mink were used. Ten dark and 10 sapphire mink were started on each of the following treatments: (1) control ranch ration (table 1); (2) basal semipurified mink ration with no added vitamin K (table 2); and (3) basal semipurified mink ration plus 7 gm of menadione sodium bisulfite (MSB)[6] per ton of feed. After 28 days, the whole blood prothrombin times were obtained with the results shown in table 3.

Received for publication January 28, 1961.

[1] Journal art. no. 2743 Michigan Agricultural Experiment Station.

[2] This work was supported in part by a grant from Abbott Laboratories, North Chicago, Illinois.

[3] Presented by the senior author in partial fulfillment of the Ph.D. degree.

[4] Present address: U.S.D.A. Agr. Res. Service, Beltsville, Maryland.

[5] Difco EE100 was used as a source of thromboplastin for the chicks and Warner-Chilcott Simplastin was used for the mink.

[6] Abbott Laboratories Klotogen F containing 63% MSB (U.S.P.) was added to provide specified amounts of vitamin K activity.

TABLE 1

Composition of control ranch ration[1]

	%
Horse meat	26.7
Ocean whiting	26.6
Beef tripe	26.7
Beef liver	5.0
Cereal[2]	15.0
Total	100.0

[1] Sufficient water added to make a hamburger consistency.

[2] Cereal 1007, Kellogg Company, Battle Creek, Michigan.

TABLE 2

Composition of semipurified basal mink ration[1]

	%
Vitamin-free casein[2]	32
Stripped lard[3]	14
Cod liver oil	1
Cellulose[4]	5
Salts[5]	4
Choline chloride (70%)	1
Sucrose	43
Vitamin supplement	—[6]
Total	100

[1] Water added to produce a thick syrup consistency for mink. Ration fed dry to chicks.

[2] Vitamin-Free Casein, Nutritional Biochemical Corporation, Cleveland.

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

[4] Solka-Floc, Brown Company, Berlin, New Hampshire.

[5] Phillips and Hart ('35), cobalt chloride, 0.005%, added.

[6] Vitamins and amino acids in milligrams per 100 gm of feed added at the expense of sucrose: arginine·HCl, 500.0; DL-methionine, 300.0; thiamine·HCl, 0.5; pyridoxine·HCl, 0.5; riboflavin, 1.0; Ca pantothenate (dextrorotatory), 3.6; nicotinic acid, 5.0; *i*-inositol, 50.0; *p*-aminobenzoic acid, 100.0; folic acid, 0.2; biotin, 0.05; α-tocopherol, 8.0; vitamin B_{12} 0.015; and BHT (butylated hydroxytoluene), 20.0.

These data show that there were no significant differences in whole blood prothrombin times due to treatment or genetic strain. Three mink receiving the purified ration without added K were continued for two additional weeks with no appreciable increase in clotting time; their average clotting times being 17.3 seconds at the end of 4 weeks and 18.3 seconds at the end of 6 weeks.

It is evident, therefore, that mink require no more vitamin K than was present in the basal semipurified ration without vitamin K supplementation. To ascertain this value, a chick assay test was conducted on the semipurified ration which had been fed to the mink. A total of 100 male Single Comb White Leghorn chicks were allocated equally to the following: lot 1, the mink semipurified basal ration; lot 2, vitamin K-free assay ration (Quick, '57); and lots 3, 4 and 5, vitamin K-free assay ration plus 22.5, 90 and 360 mg of MSB (USP) per ton, respectively.

TABLE 4

Whole blood prothrombin times of 9- to 10-day-old chicks receiving mink basal, chick basal and chick basal plus graded levels of menadione sodium bisulfite (MSB) from hatching time[1]

Ration	Whole blood prothrombin time in seconds
Mink semipurified basal	67.5 ± 6.8[2]
Vitamin K-free chick basal	93.9 ± 9.4
Chick basal + 22.5 mg/ton (MSB)	53.0 ± 5.6
Chick basal + 90 mg/ton (MSB)	26.5 ± 1.5
Chick basal + 360 mg/ton (MSB)	22.1 ± 1.1

[1] Ten chicks/treatment.

[2] Standard error.

TABLE 3

Whole blood prothrombin times of mink after receiving the specified experimental rations for 28 days

Rations	Type of mink	No. mink	Mean clotting time in seconds
Control ranch	Dark	10	17.8 ± 0.36[1]
	Sapphire	10	16.8 ± 0.42
Semipurified without vitamin K	Dark	10	18.1 ± 0.37
	Sapphire	10	17.2 ± 0.28
Semipurified plus 7 gm/ton vitamin K (MSB)	Dark	10	17.4 ± 0.33
	Sapphire	10	17.6 ± 0.43

[1] Standard error of the mean.

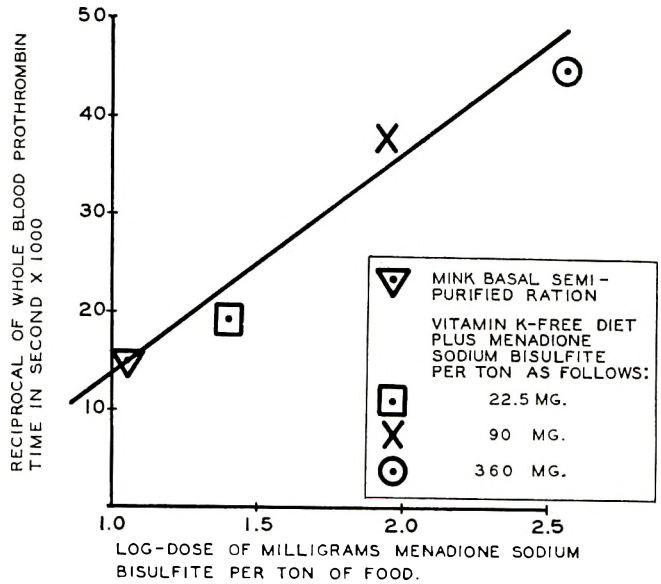

Fig. 1 Determination of vitamin K (menadione sodium bisulfite) level of mink basal semipurified ration using chick assay.

TABLE 5

Effect of terramycin, aureomycin or sulfaquinoxaline (SQ) alone and in the presence of vitamin K on whole blood prothrombin times of mink[1]

Lot	Ration	No. mink	Mean whole blood prothrombin time in seconds
1	Mink ranch diet	20	18.9 ± 0.15[2]
2	Terramycin, 400 gm/ton	10	19.6 ± 0.27
3	Aureomycin, 400 gm/ton	10	19.3 ± 0.35
4	SQ, 0.05%	10	21.6 ± 1.02
5	SQ, 0.06%	10	26.6 ± 3.0
6	SQ, 0.05% + vitamin K (1 gm/ton MSB)	10	21.2 ± 9.8
7	SQ, 0.06% + vitamin K (10 gm/ton MSB)	10	21.2 ± 0.83

Comparisons of means by analysis of variance and Duncan's multiple range and multiple F tests: (values not underscored are significantly different at $P < 0.01$).

Lot	1	3	2	6	7	4	5
Mean	18.9	19.3	19.6	21.2	21.2	21.6	26.6

[1] Each group 8 to 9 days on experiment; half of group was bled on each day.
[2] Standard error.

Using the method of Almquist and Klose ('39) of plotting mean reciprocal prothrombin times against the logarithms of the vitamin K dosage, this test showed that the semipurified ration fed the mink contained 13 mg of MSB (USP) per ton (fig. 1). This demonstrates that adult mink need less vitamin K than 13 mg per ton of feed, air dry basis. A level as low as this would be virtually impossible to attain in a practical ranch ration.

Experiment 2. In another phase of the investigation, the effects of certain medicaments and drugs upon blood clotting time were studied. When sulfaquinoxaline was fed to mink as a coccidiostat, it upset the normal blood clotting process and caused severe hemorrhages in many cases

(Hartsough and Gorham, '60). Since sulfaquinoxaline affects the bacterial flora of the gut so that vitamin K is not produced (Kornberg et al., '44), aureomycin and terramycin which are also used in mink therapy were investigated. Results of this study are shown in table 5.

Terramycin or aureomycin at levels of 400 gm per ton did not significantly increase blood clotting time. Sulfaquinoxaline at levels of 0.05% or more significantly increased clotting time. A level of 10 gm MSB (USP) per ton decreased the clotting time when administered in the presence of 0.06% of sulfaquinoxaline, but did not lower it to the normal range. MSB fed at the rate of 1 gm per ton did not significantly decrease blood clotting time in the presence of 0.05% sulfaquinoxaline. In preliminary studies, levels of sulfaquinoxaline of 0.1% or more caused death by cardiac tamponade (acute compression of heart due to effusion of blood into pericardium) in about one half of the mink from which blood was drawn by heart puncture.

SUMMARY AND CONCLUSIONS

Experiments were conducted to ascertain: (1) whether normal adult mink require vitamin K; (2) whether dark mink differ in this respect from sapphire mink which contain the Aleutian gene; and (3) whether sulfaquinoxaline or certain antibiotics affect blood clotting time.

The dietary requirement of normal adult mink was established as being less than 13 mg of menadione sodium bisulfite (USP) per ton, or 6.5 μg per pound of feed. Practical ranch rations would contain much higher levels of vitamin K activity.

The mean whole blood prothrombin time of sapphire mink (16.8 ± 0.4 seconds) was similar to that of dark mink (17.8 ± 0.4 seconds).

Sulfaquinoxaline fed for 6 to 8 days at the 0.05% level or higher significantly increased the whole blood prothrombin times.

Menadione sodium bisulfite (USP) at a level of 10 gm per ton of food (fed as Klotogen F) reduced the blood clotting time of mink fed sulfaquinoxaline at a level of 0.06%.

Addition of aureomycin and terramycin at a level of 400 gm per ton of food when fed for 6 to 8 days did not significantly increase whole blood prothrombin times.

Mink fed a semipurified ration had comparable blood clotting times to those fed a typical ranch ration.

LITERATURE CITED

Almquist, H. J., and A. A. Klose 1939 Determination of the antihemorrhagic vitamin. Biochem. J., *33:* 1055.

Frost, D. V., H. D. Perdue and H. C. Spruth 1956 Vitamin K activity of menadione sodium bisulfite in chickens. J. Nutrition, *59:* 181.

Hartsough, G. R., and J. Gorham 1960 Sanitation and Health. The Blue Book of Fur Farming, Editorial Service Company, Milwaukee, Wisconsin.

Helmboldt, C. F., and E. L. Jungherr 1958 The pathology of Aleutian disease in mink. Am. J. Vet. Res., *19:* 212.

Kainer, R. A. 1954 The gross anatomy of the digestive system of the mink. Ibid., *15:* 82.

Kornberg, A., F. S. Daft and W. H. Sebrell 1944 Mechanism of production of vitamin K deficiency in rats by sulfonamides. J. Biol. Chem., *155:* 193.

Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

Quick, A. J. 1957 Hemorrhagic Diseases. Lea and Febiger, Philadelphia, Pennsylvania.

Wood, A. J. 1956 Time of passage of food in mink. Black Fox Mag. Mod. Mink Breeder, *29:* 9.

Effect of Methionine Deficiency on the Utilization of Energy by the Chick

L. B. CAREW, JR.[1] AND F. W. HILL[2]
Department of Poultry Husbandry, Cornell University, Ithaca, New York

The wide use of soybean oil meal as a major source of protein in animal nutrition and its moderate deficiency in sulfur amino acids, has led to extensive studies of methionine supplementation of diets based on soybean protein. Investigations of the requirement of the chick for methionine and total sulfur amino acids have been reviewed by Almquist ('52) and Hill ('53). More recently, the effects of dietary energy level on the methionine requirement of the chick have been studied (Baldini and Rosenberg, '55; Rosenberg and Baldini, '57; Nelson et al., '60). Although the effects of amino acid deficiences on growth and gross efficiency of diet utilization (weight gain per unit of diet consumed) have been widely studied, little is known about the effects of methionine deficiency on the pattern of energy utilization by the chick. Because of the marked changes in growth and food intake that occur with amino acid deficiencies, it is generally assumed that changes in efficiency of energy utilization occur also.

One interesting aspect of studies with methionine is the observation in several laboratories (Saxena and McGinnis, '52; Slinger et al., '53; Nelson et al., '60) that a higher level of dietary methionine is required to produce maximal gross efficiency than is necessary to promote maximum growth. This work is interpreted to show that chicks overeat when fed a diet slightly deficient in methionine, and although maximal growth is attained, the additional feed required to reach maximal growth results in a decreased gross efficiency of feed utilization. Possible explanations for the decreased efficiency of feed utilization and the disposal of the additional food consumed appear to be as follows: (1) decreased digestibility of the diet; (2) decreased net energy yield of the diet; (3) increased heat production; and (4) greater storage of energy in the form of adipose tissue. The study to be described was conducted to determine which of these mechanisms are involved.

EXPERIMENTAL

The methionine-deficient semipurified basal diet used in this experiment is presented in table 1. The major source of protein was commercial, solvent-extracted dehulled soybean meal. The diet was supplemented with sources of unidentified factors and contained adequate levels of all nutrients known to be required by the chick with the exception of methionine. It contained 22.8% of protein and 315 Cal. of metabolizable energy per 100 gm

TABLE 1
Composition of basal diet

	%
Glucose[1]	43.8
Soybean meal (50% crude protein)	39.0
Corn oil	5.0
Fish solubles (50% solids)	2.0
Corn distillers' dried solubles	4.0
Glycine	1.0
Minerals and vitamins[2]	4.2
Chromium oxide mix[3]	1.0
	mg/kg
Chlortetracycline HCl	5.5

[1] Cerelose, Corn Products Refining Company, New York.

[2] Supplies in mg/100 gm of diet: dicalcium phosphate, 3200; iodized salt, 300; manganese sulfate, 17; potassium iodide, 0.002; choline chloride, 140; niacin, 3; Ca-D-pantothenate, 2.5; riboflavin, 1; pyridoxine·HCl, 1; thiamine·HCl, 0.5; folacin, 0.2; menadione, 0.1; biotin, 0.04; *d*-α-tocopheryl acetate, 2.2; and vitamin B_{12}, 1 μg; stabilized vitamin A, 1000 I. U.; vitamin D_3, 150 I. C. U.

[3] Contains 30% of chromium oxide in wheat flour.

Received for publication January 16, 1961.

[1] Present address: The Rockefeller Foundation, Apartado Aereo 58-13, Bogota, Colombia, S. A.

[2] Present address: Department of Poultry Husbandry, University of California, Davis.

(air-dried basis). Using values established by microbiological assay in this laboratory, the basal diet was estimated to contain 0.29% of methionine and 0.60% of total sulfur amino acids.

Male Rhode Island Red X Barred Plymouth Rock chicks were kept in electrically heated, thermostatically controlled battery brooders with raised wire floors. They were reared to two weeks of age with a stock diet adequate in all nutrients (diet E9 of Hill et al., '60). At this time, 9 lots of 10 chicks each were selected from a group of 350 in a manner similar to that of McKittrick ('47) based on weight and rate of gain. One lot of 10 chicks was killed to provide data on initial body composition. Remaining lots of chicks were allocated to 4 treatments, duplicate lots being used per treatment. The experimental period was from 14 to 28 days of age.

Two diets were used: the methionine-deficient basal diet, and the basal diet with 0.34% of added methionine. Each diet was fed ad libitum. Daily, at approximately the same time each day, the 24-hour consumption of the lots fed each diet ad libitum was determined, and 60% of this average feed intake was then given to the chicks fed the respective restricted regimen. Feed wastage was determined by recovering it in shallow screened trays placed beneath the feeders. Water was supplied ad libitum.

For analysis of body composition, the chicks were killed at the termination of the experiment by dislocation of the neck without loss of blood, the intestinal contents were removed, and the entire carcasses were frozen, ground and dried from the frozen state. For determination of metabolizable energy, 0.3% of chromium oxide was added to the diet as an index substance and excreta were collected at 24-hour intervals during the last 4 days of the experiment. The methods used for carcass analysis and metabolizable energy determinations were those described by Hill and Anderson ('58).

A slight modification of the method described by Fraps ('46) was used to estimate productive (net) energy. It was partly for this purpose that chicks were fed at two levels of nutrition. The modifications were: (1) measurements were made using 10 chicks as a treatment unit rather than individuals, and (2) a value of 4.34 Cal. per gm for body protein was used in calculating energy gain, rather than 5.66 Cal. The latter change takes into account the energy of catabolized protein which is lost as urinary products and cannot be used by the chick for productive purposes. The energy coefficient for protein was reduced by the combustible energy of an equivalent amount of uric acid (from 5.66 to 4.34 Cal. per gm). This adjustment is similar to the correction for nitrogen retention used in the measurement of metabolizable energy. Its use made the productive energy values more directly comparable to metabolizable energy, and provided a more rational basis for relating tissue energy gains to metabolizable energy consumption.

Gains in protein and fat during the experimental period were determined from the difference between composition of the lots at 28 days of age and that of the group sacrificed at 14 days of age.

Analysis of variance and comparisons of single degrees of freedom (Snedecor, '56) were used for determining statistical significance.

RESULTS AND DISCUSSION

The results obtained for weight gains, feed consumption and gross efficiency of feed utilization are presented in table 2. The degree of methionine deficiency established in this experiment had no significant effect on growth rate when the diets were fed ad libitum. A significant decrease ($P < 0.05$) in gross efficiency, however, was observed with the deficiency of methionine, due mainly to an increase in feed consumption. Groups 3 and 4 fed the two diets at restricted levels showed the same relative differences as groups 1 and 2. These data were in agreement with those discussed previously, showing that the methionine requirement for maximal growth was not adequate for maximal efficiency.

The results noted for the metabolizable energy values of the diet are presented in table 3. No significant difference was observed in metabolizable energy values, and the small difference between treatments 1 and 2 favored the methionine-deficient diet. Therefore, the data in table 3 clearly show that methionine deficiency

TABLE 2

Effect of methionine on weight gain, feed consumption and gross efficiency[1]

	Treatment	Weight gain 2-4 weeks	Feed consumed (dry matter)	Gain/unit feed intake
		gm	*gm*	
1	Basal diet, ad libitum	228	444	0.513
		226	431	0.523
		227	*437*	*0.518*
2	Basal diet + 0.34% methionine, ad libitum	230	411	0.560
		235	419	0.562
		233	*415*	*0.561*
3	Basal diet, feed intake restricted to 60% of no. 1	127	263	0.483
		122	258	0.475
		125	*261*	*0.479*
4	Basal diet + 0.34% methionine, feed intake restricted to 60% of no. 2	132	248	0.533
		123	252	0.489
		128	*250*	*0.511*

[1] Italic data are the averages of duplicate lots.

TABLE 3

Effect of methionine on metabolizable energy, productive energy and heat production[1]

	Treatment	1 Metabolizable energy	2 Productive energy	1 − 2 Estimate of heat increment
		Cal./gm diet	*Cal./gm diet*	*Cal./gm diet*
1	Basal diet, ad libitum	3.41	1.79	1.62
		3.39	1.85	1.54
		3.40	*1.82*	*1.58*
2	Basal diet + 0.34% methionine, ad libitum	3.34	1.71	1.63
		3.31	1.76	1.55
		3.33	*1.74*	*1.59*
3	Basal diet, feed intake restricted to 60% of no. 1	3.41		
		3.36		
		3.39		
4	Basal diet + 0.34% methionine, feed intake restricted to 60% of no. 2	3.38		
		3.42		
		3.40		

[1] Italic data are the averages of duplicate lots.

did not impair the chick's ability to metabolize dietary energy. Since any change in digestibility of the diet would be reflected in metabolizable energy measurements, the decreased efficiency observed with the methionine-deficient diet cannot be attributed to a lower digestibility of this diet. Pisano et al. ('59) have studied the effect of a methionine deficiency on nitrogen absorption from the intestinal tract of the chick. Although these workers observed that chicks fed a methionine-deficient diet absorbed nitrogen more rapidly in the first part of the intestinal tract, no significant differences were observed in total nitrogen absorption between chicks receiving diets adequate and deficient in methionine. Our results, therefore, are in agreement with the concept that a methionine deficiency does not affect the total absorption of dietary nutrients.

Also shown in table 3 are productive energy values as determined by carcass analysis technique. A difference of approximately 5% was observed between the two diets. Since greater variability

than this is usually encountered in productive energy measurements, this difference was not considered meaningful. Moreover, the numerical difference in values was in favor of the methionine-deficient diet, showing that the lower efficiency of feed utilization associated with this diet could not be due to decreased net energy yield. The difference between metabolizable energy and productive energy gives an estimate of heat increment per gram of diet consumed. These differences were almost identical, indicating that there was no difference in heat increment between the methionine-deficient diet and the methionine-adequate diet (table 3).

Differences in carcass composition between treatments were observed (table 4). Chicks maintained with the methionine-deficient diet showed significantly less nitrogen retention. It is well known that amino acid deficiencies and imbalances decrease nitrogen retention. Data on carcass composition also showed that the methionine deficiency resulted in a greater fat deposition, both as a percentage of the carcass and in absolute amount. Although the deficiency of this amino acid limited protein synthesis, fat deposition was evidently not impaired.

The deficient chicks stored more energy than the methionine-adequate chicks, (table 5). Although the lack of methionine caused less energy to be stored as protein, this was more than compensated for by the increased retention of energy as fat, with the result that the methionine-deficient chicks on treatment 1 had significantly greater total energy gains than those fed the adequate diet.

The relationship between energy intake expressed as metabolizable calories, and the total gain in carcass calories for the two-week experimental period is shown in figure 1. The regression lines for the two treatments were established. For the methionine-deficient lots (treatments 1 and 3), the line was described by the equation $Y = 0.379X - 163$. For the lots fed the methionine-supplemented basal diet (treatments 2 and 4), this equation was $Y = 0.414X - 205$. No significant difference between regressions was noted, and all points fitted a single line having a correlation coefficient of 0.99. This line describes the distribution of ingested calories between tissue gains and heat production in relation to energy intake. The closeness of fit of all points to a single linear regression indicates that all lots, regardless of methionine adequacy, utilized their caloric intake with equal efficiency and that the differences in energy gain were due entirely to differences in energy consumption. Such an interpretation agrees well with the similarity of values obtained for heat increment per gram of diet as presented in table 3.

TABLE 4

Effect of methionine on carcass composition and body tissue gains[1]

	Treatment	Protein[2]	Fat[2]	Moisture	Gain in protein	Gain in fat
		%	%	%	*gm*	*gm*
1	Basal diet, ad libitum	60.4	29.2	69.3	43.0	20.9
		58.8	30.8	68.9	42.4	23.3
		59.6	*30.0*	*69.1*	*42.7*	*22.1*
2	Basal diet + 0.34% methionine, ad libitum	63.0	26.8	69.5	45.1	18.0
		62.4	26.9	70.5	45.3	18.4
		62.7	*26.9*	*70.0*	*45.2*	*18.2*
3	Basal diet, feed intake restricted to 60% of no. 1	64.4	24.1	71.6	22.4	5.9
		63.7	25.5	71.0	23.1	7.3
		64.1	*24.8*	*71.3*	*22.8*	*6.6*
4	Basal diet + 0.34% methionine, feed intake restricted to 60% of no. 2	68.3	20.9	69.3	25.3	3.5
		66.9	21.1	72.6	26.0	4.2
		67.7	*21.0*	*71.0*	*25.7*	*3.9*

[1] Italic data are the averages of duplicate lots.
[2] Expressed as percentage of moisture-free carcass.

TABLE 5

Effect of methionine on energy utilization[1]

	Treatment	Intake of metabolizable energy	Protein gained	Fat gained	Total gain
		Cal./chick	*Cal./chick*	*Cal./chick*	*Cal./chick*
1	Basal diet, ad libitum	1514	187	195	382
		1461	184	218	402
		1488	*185*	*207*	*392*
2	Basal diet + 0.34% methionine, ad libitum	1372	196	168	364
		1387	197	172	369
		1380	*196*	*170*	*366*
3	Basal diet, feed intake restricted to 60% of no. 1	897	97	55	152
		867	99	68	168
		882	*98*	62	*160*
4	Basal diet + 0.34% methionine, feed intake restricted to 60% of no. 1	838	110	33	143
		862	113	39	152
		850	*111*	*36*	*147*

[1] Italic data are the averages of duplicate lots.

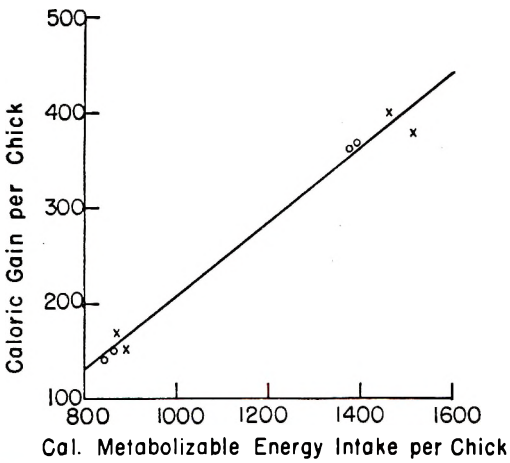

Fig. 1 Linear relationship between metabolizable energy intake and tissue caloric gains for chick fed an adequate diet (○) and one deficient in methionine (×).

This relationship does not imply that all chicks retained the same proportion of ingested calories as tissue calories, since the line does not pass through the origin. The interpretation of this linear relationship is based on the view that all points fitting the line established by chicks fed an adequate diet represent treatments which did not alter overall energy utilization. Any treatments which decrease the efficiency of energy utilization would produce points significantly below this control line, and any increase in efficiency of energy utilization would produce a response above the line. Furthermore, when treatments lie on the control line, any differences in energy gains can be attributed to differences in energy intake.

The validity of considering the relationship between food intake and energy gain to be linear may be questioned, since the data from this experiment are not sufficient to establish the nature of this relationship. Previous studies in this laboratory (Hill and Anderson, '58) have shown such a linear relationship for the combined data of several experiments in which a broad range of levels of food intake (from 30 to 100% of ad libitum) was used. In other unpublished experiments we have found that energy gains of chicks fed graded levels of food intake over short (14-day) periods closely follow a linear regression which can be accurately established for a given experiment. The slope of the regression varies between experiments, undoubtedly due to differences in environmental conditions which influence the partition of energy between gains and heat production.

From the regression equation calculated for the control chicks (treatments 2 and 4), the average expected gain for the chicks in treatment 1 receiving the methionine-deficient basal diet ad libitum was 411 Cal. The expected gain is slightly higher than that observed. Therefore, although the data of this experiment show that the methionine-deficient chicks made

greater carcass gains because of their extra intake of metabolizable calories, the small difference between expected and observed gains suggests that there was a slight decrease in the efficiency of energy utilization. This effect, however, was so small that it was not detected in the measurement of productive energy and heat increment, or by the partitioning of energy intake between tissue gains and heat production.

The decreased gross efficiency observed for methionine-deficient chicks in this experiment may then be explained as follows. The methionine-deficiency increased food intake, and more metabolizable energy was consumed. There was also an increased synthesis of fat concomitant with a decrease in nitrogen retention, with the net result that more calories were deposited as fat and less as protein. Therefore, there was a greater concentration of calories per gram of body gain. Although the increased energy consumed by the methionine-deficient chicks was metabolized with normal efficiency, it did not appear as additional weight gain because of the change in body composition.

SUMMARY

The effects of a moderate deficiency of sulfur amino acids in the diet of young chicks on energy utilization were studied. The deficient diet (sulfur amino acids 2.6% of protein) did not significantly retard growth, but produced lowered gross efficiency of gains as compared with the adequate diet (sulfur amino acids 4% of protein). No differences in efficiency of energy metabolism were observed based on determinations of metabolizable energy, productive energy, heat production and tissue gains. Chicks fed the methionine-deficient diet, however, had greater gains in tissue fat but lower gains of protein. As a consequence, the higher caloric intake of chicks fed the methionine-deficient diet did not produce additional weight gain. The differences in gross efficiency and in body composition produced by the methionine-deficient and methionine-adequate diets were shown to be due entirely to the differences in food intake.

ACKNOWLEDGMENT

This work was supported in part by a research grant of the Monsanto Chemical Company, St. Louis, Missouri.

LITERATURE CITED

Almquist, H. J. 1952 Amino acid requirements of chickens and turkeys. A review. Poultry Sci., *31:* 966.

Baldini, J. T., and H. R. Rosenberg 1955 The effect of productive energy level of the diet on the methionine requirement of the chick. Ibid., *34:* 1301.

Fraps, G. S. 1946 Composition and productive energy of poultry feeds and rations. Texas Agr. Exp. Station Bull., p. 678.

Hill, F. W. 1953 New information on lysine and methionine requirements of chicks. Proc. Cornell Nutrition Conf. for Feed Manufacturers, p. 44.

Hill, F. W., and D. L. Anderson 1958 Comparison of metabolizable energy and productive energy determinations with growing chicks. J. Nutrition, *64:* 587.

Hill, F. W., D. L. Anderson, Ruth Renner and L. B. Carew, Jr. 1960 Studies of the metabolizable energy of grain and grain products for chickens. Poultry Sci., *39:* 573.

McKittrick, D. S. 1947 The selection of chicks for growth experiments and the evaluation of growth. Growth, *11:* 89.

Nelson, T. S., R. J. Young, R. B. Bradfield, J. B. Anderson, L. C. Norris, F. W. Hill and M. L. Scott 1960 Studies on the sulfur amino acid requirement of the chick. Poultry Sci., *39:* 308.

Pisano, J. J., C. M. Paine and M. W. Taylor 1959 The effect of methionine deficiency on nitrogen absorption from the intestinal tract of chickens. J. Nutrition, *67:* 213.

Rosenberg, H. R., and J. T. Baldini 1957 Effect of dietary protein level on the methionine-energy relationship in broiler diets. Poultry Sci., *36:* 247.

Saxena, H. C., and J. McGinnis 1952 Effect of DL-methionine on feed utilization by chicks and turkey poults, circular 197. Washington Agr. Exp. Sta., Pullman, Washington.

Slinger, S. J., W. F. Pepper and D. C. Hill 1953 Value of methionine supplementation of chick and poult diets containing a high percentage of wheat. Poultry Sci., *32:* 573.

Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State College Press, Inc., Ames.