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BIOLOGICAL AVAILABILITY OF TRYPTOPHAN[1]

J. D. GUPTA AND C. A. ELVEHJEM

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

(Received for publication December 18, 1956)

The nutritive value of a protein depends not only upon its content and balance of essential amino acids but also upon the availability of the amino acids to the animal. Several workers (Hankes et al., '48; Riesen et al., '47; Denton and Elvehjem, '53; Mauron et al., '55) have studied the rate and extent of liberation of amino acids from different proteins during digestion *in vitro* using proteolytic enzymes and measuring the liberated amino acids chemically or microbiologically. These *in vitro* digestion studies may not give a true index of the availability to the intact animal where a multiple enzyme system acts during the process of digestion and other factors such as the rates of absorption of the amino acids and their simultaneous appearance at the sites of protein synthesis influence the determination of availability.

The availability of tryptophan from proteins for growth of chicks (Schweigert, '48) and of lysine (Schweigert and Guthneck, '53) and methionine (Schweigert and Guthneck, '54) for the rat has been determined.

The investigation reported in this paper deals with the determination of the availability of tryptophan from various

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Livestock and Meat Board, Chicago, Illinois. The authors are indebted to Dr. A. M. Swanson of the Department of Dairy and Food Industry for the milk solids and to Dr. A. E. Harper for help in the preparation of the manuscript. The crystalline vitamins were provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

proteins using growth of weanling rats as the index of availability. The unavailable tryptophan in the feces has also been determined microbiologically and the results obtained with two methods compared.

EXPERIMENTAL

Male weanling rats of the Holtzman strain weighing about 40 to 50 gm were used throughout the study. They were divided into similar groups of 6 animals each and were maintained in individual suspended cages with screen bottoms. Food and water were given ad libitum and the animals were weighed weekly.

The percentage composition of the basal diet was: casein, 5; gelatin, 10; zein, 10; L-lysine·HCl, 0.2; L-histidine·HCl, 0.15; DL-methionine, 0.3; DL-threonine, 0.2; salts IV (Hegsted et al., '41), 4; corn oil, 5; vitamin mixture in sucrose, 0.25; choline chloride, 0.15 and dextrin to make 100. The 0.25 gm of vitamin mixture contained in milligrams; thiamine·HCl, 0.5; riboflavin, 0.5; niacin, 2.5; calcium pantothenate, 2.0; pyridoxine, 0.25; biotin, 0.05; folic acid, 0.02; vitamin B_{12}, 0.002 and inositol, 10.0. Two drops of halibut liver oil fortified with vitamins E and K were administered orally each week. The only limiting factor in the protein portion of this diet was tryptophan.

When the basal diet, which contained 0.06% of tryptophan, was supplemented with graded levels of L-tryptophan growth responses proportional to the level of tryptophan supplementation were obtained. From these values a standard curve was drawn by plotting the average gain in grams against the amount of L-tryptophan supplement. The growth of rats receiving the basal diet supplemented with different levels of a protein of known tryptophan content was determined. The amount of L-tryptophan corresponding to this growth as read from the standard curve was taken as the amount of tryptophan available from that contained in the

protein supplement. The percentage availability was calculated as follows:

$$\text{percentage availability} = \frac{\text{amount of tryptophan available as read from standard curve}}{\text{amount of tryptophan as determined microbiologically in the protein supplement}} \times 100.$$

The protein and tryptophan supplements were added at the expense of dextrin as it was found in preliminary experiments that such a procedure gave results almost identical with those obtained when the proteins were fed at the expense of zein (very low in tryptophan) or gelatin (devoid of tryptophan).

The tryptophan content of the proteins, the diets and feces were determined according to the method of Kuiken, Lyman and Hale ('47). Samples were hydrolyzed with 5 N NaOH and the hydrolysates were assayed microbiologically using *S. faecalis*.

The food consumption of each rat was recorded. By placing the food cup in a larger container losses from spillage were kept to a minimum.

Preparation of beef and pork samples. Fat and bones were removed from the pork and beef cuts as completely as possible and then they were roasted in an oven at 400 to 450°C. for 4 to 5 hours. The liquid fat was drained off, any remaining bone was removed and the meat was ground in a meat grinder. The ground meat was dried at 50 to 60°C. for three to 4 days and then extracted with Skelly Solve B in a Soxhlet extractor for several hours. The extracted meat was again dried and was ground in a Wiley mill with a medium mesh screen. The beef and pork samples prepared in this way contained about 90.5 and 91.4% of protein respectively.

RESULTS

The growth responses obtained over a period of two weeks when graded levels of L-tryptophan were added to the basal diet are shown in table 1.

TABLE 1

Growth responses with graded levels of L-*tryphophan added to basal diet*

L-TRYPTOPHAN SUPPLEMENT TO THE BASAL DIET	GAIN/RAT [1] A	AVERAGE FOOD CONSUMED/RAT DURING EXP. PERIOD	AVERAGE GAIN/RAT/100 GM FOOD B
mg/100 gm	*gm*	*gm*	*gm*
None	16.4 ± 1.2 [2]	78	21
15	33.6 ± 2.2	116	29
30	48.4 ± 3.2	132	36.7
45	66.4 ± 4.0	151	43.9

[1] Both A and B were plotted against the L-tryptophan supplement to get two different standard curves and the samples were read from these curves after similar conversions of the gain in weight during the whole experimental period.

[2] Mean and standard error.

Under the conditions of this experiment and with a tryptophan supplement not exceeding 0.045% a near linear relationship existed between the growth responses and the levels of L-tryptophan added. This type of standard curve, which was repeated for each set of experiments, was used to compute the availability of tryptophan from the proteins.

The availability of tryptophan from some proteins which were fed at 2% levels in the basal diet is shown in table 2.

TABLE 2

Availability of tryptophan from different proteins fed at 2% level

PROTEIN	AMOUNT OF SUPPLEMENTARY TRYPTOPHAN PRESENT IN THE DIET	AVAILABLE TRYPTOPHAN AS READ FROM STANDARD CURVE A	AVAILABILITY
	mg/100 gm	*mg*	%
Beef	20.4	20.3	99.5
Pork	22.4	20	89.3
Casein	20.4	16.5	80.9
Soybean protein [1]	16.7	14.5	87
Beef [2]	20.4	21	102.9

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

[2] Added at the expense of the gelatin whereas others were added at the expense of dextrin.

It is evident from the results that tryptophan was available to the extent of 80 to 100% from the proteins tested, the maximum being from beef proteins where it was 100% available. It was interesting to note that tryptophan was about 87% available from a commercial purified soybean protein.[2]

However, in more extensive experiments with beef and pork it was observed that the availability as measured by this procedure was influenced by total amount of tryptophan in the supplement. When the amount of tryptophan was increased either by raising the level of the protein supplement or by adding some L-tryptophan along with the protein supplement the availability values were significantly lower as shown in table 3.

There was also a wide variation in the average food consumption in different experimental groups. We, therefore, calculated the average weight gains for the groups receiving various levels of tryptophan per 100 gm of food consumed — which, in fact, is a measure of food efficiency. These values plotted against the level of tryptophan supplementation gave another standard curve. The amount of available tryptophan in each of the experimental samples was also read from this type of curve. These results are shown in tables 2, 3 and 4.

The percentage availability computed in this way is slightly higher in most cases than that obtained by the other method. However, the differences are such that for all practical purposes in a biological assay of this type they may be thought to be in fairly close agreement. There is a trend towards slightly higher availability of tryptophan from beef than from pork (table 3).

The variation of the percentage availability with the amount of tryptophan in the supplement as shown in table 3, led us to compare the availability from different proteins when fed at the same tryptophan level. It can be noted that the percentage availability from beef, pork and fibrin (not fed at the same tryptophan level) was about the same. Only egg

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

TABLE 3

Availability of tryptophan from pork and beef fed at different levels and with added L-*tryptophan*

(Separate standards were run with each experiment)

SUPPLEMENT TO BASAL DIET	SUPPLEMENTARY TRYPTOPHAN IN THE DIET	GAIN/RAT A	AV. FOOD CONSUMED/RAT	AV. GAIN/RAT/100 GM FOOD B	AVAILABILITY CALCULATED FROM	
					A	B
	mg/100 gm	*gm*	*gm*	*gm*	%	%
			Experiment no. 1			
Beef, 2%	20.4	39	118	31	98	105.4
Beef, 3%	30.6	49	136	36	96.4	93.7
Beef, 2% + 0.015% L-tryptophan [1]	35.4	54.6	143	38.2	85.8	88.2
Beef, 2% + 0.030% L-tryptophan [1]	50.4	66	145	45.4	70.1	77.9
DL-Tryptophan, 0.040%	40	58	141	41.2	93.8	96.2
			Experiment no. 2			
Pork, 2%	22.4	37.4	115	32.4	89.3	107.1
Pork, 3%	33.6	42.6	115	37	72.9	92.2
Pork, 2% + 0.020% L-tryptophan [1]	42.4	55.2	143	38.6	66.9	69.2

[1] Availability from protein was calculated on the assumption that the added L-tryptophan was completely available.

albumin showed a slightly lower availability. Further, for each of the proteins the percentage availability values, calculated in both ways, were in fairly close agreement with each other.

We have also compared the availability of tryptophan in three differently processed milk solid preparations fed at the same tryptophan levels. Here again, as shown in table 4, 94 to 98% of the tryptophan was available from each product using either type of computation. On the other hand, with weight gain as the criterion a lower percentage availability, namely 78%, was found for the roller dried powder.

The percentage utilization of DL-tryptophan by rats was also determined by the same method and was found to be 93.8 and 96.2 by two different methods of computation (table 3), in close agreement with the values obtained by Osterling and Rose ('52).

The amounts of unavailable tryptophan in the feces of some of the experimental groups of table 4 were determined by microbiological assay and these results are shown in table 5. Here again the amounts of unavailable tryptophan with protein supplements of beef, pork, egg albumin and fibrin were low suggesting a high percentage of availability. This is in fairly good agreement with the values reported in table 4.

TABLE 4

Availability of tryptophan from (1) different proteins and (2) some milk solids fed at equal tryptophan level

SUPPLEMENTS TO BASAL DIET	AMT. OF SUPPLEMENTARY TRYPTOPHAN IN THE DIET	GAIN/RAT A	AV. FOOD CONSUMPTION/RAT	AV. GAIN/RAT/100 GM FOOD B	AVAILABILITY CALCULATED FROM	
					A	B
	mg/100 gm	*gm*	*gm*	*gm*	*%*	*%*
Pork, 2%	22.5	42.6	138	30.8	91.1	93.3
Beef, 2.2%	22.5	43.6	141	31	93.3	95.6
Egg albumin, 1.9%	22.5	40.6	134	30.2	84.4	86.6
Fibrin,[1] 0.91%	29.1	52	154	33.6	92.7	89.4
Roller dried non-fat milk powder, 5.5%	25	43.4	128	32.4	78	96
Spray dried non-fat milk powder, 5.5%	25	48	150	32	98	94
Spray dried non-fat milk solid grains (instant type), 5.5%	25	48	147	32.6	98	98

A and B — implication as in table 3.

[1] Not fed at the same tryptophan level. Fibrin was fed at this level from the tryptophan value obtained by alkaline hydrolysis of fibrin for 10 hours as in the case of other proteins. However, a higher tryptophan value was obtained where fibrin was hydrolyzed for 18 hours which has been used in subsequent calculations. (Kuiken, Lyman and Hale, '47).

TABLE 5

Determination of unavailable tryptophan in the feces

GROUP	TOTAL FOOD CONSUMED	TOTAL SUPPLEMENTARY TRYPTOPHAN IN FOOD CONSUMED	X [1] TOTAL TRYPTOPHAN IN THE FECES	Y AMT. UNAVAILABLE TRYPTOPHAN FROM BASAL PROTEINS + ENDOGENOUS TRYPTOPHAN	X-Y UNAVAILABLE TRYPTOPHAN FROM SUPPLEMENT	% AVAILABLE
	gm	*mg*	*mg*	*mg*	*mg*	
Basal	525	none	81.5	81.5		
Basal + 2% pork	825	185.6	142	128.7	13.3	92.8
Basal + 2.2% beef	848	191.2	140	132.6	7.4	96.1
Basal + 1.9% egg albumin	801	180	138.4	125	13.4	92.5
Basal + 0.91% fibrin	925	269.2	169.1	144.3	24.8	90.8

[1] X — Found by microbiological assay of a representative aliquot of the feces collected for the whole group and for the entire experimental period.

Y — Calculated as follows:

$$= \frac{\text{Total amount of tryptophan in the feces of basal group}}{\text{Total food consumed by the basal group}} \times \text{Total food consumed by the experimental group.}$$

$$\% \text{ Tryptophan available} = \frac{\text{Total tryptophan in supplement} - \text{unavailable tryptophan from supplement} \times 100}{\text{Total tryptophan in supplement}}$$

Total tryptophan in supplement

DISCUSSION

The growth difference of 25 to 28 gm between the lowest and highest levels of tryptophan used for the standard curves gave a good range over which to measure availability. However, since very little additional tryptophan was required to give values towards the top of the linear portion of the curve the number of levels that could be included was limited. With only 4 points on the linear portion of the curve any deviation from linearity in one of the groups could lead to an error in the slope of the curve and hence in the estimation

of tryptophan availability. Despite this limitation the results computed from the two different types of standard curves were in good agreement and replicate determinations also showed good agreement.

That tryptophan was the only limiting factor in the basal diet is shown by the fact that with tryptophan supplementation only, the growth rates increased to 33 to 35 gm/rat/wk. which is comparable to the growth obtained with an adequate purified diet. Further, there is no possibility of any type of imbalance being produced by the addition to the basal diet of the protein tested because of the low levels at which the proteins were added and also because none of the other amino acids in the diet were in near limiting amounts. The high availability values obtained with the proteins tested in our experiments point to the fact that no imbalance of any type has been produced.

The possibility that the high availability figures may be due to an increased response to the entire protein supplement seems unlikely. This is supported by our observation (table 1) that the same percentage availability from beef was found irrespective of whether the protein supplement was added at the expense of gelatin or dextrin. If the protein had given a response in addition to that due to tryptophan, then the higher level of protein supplementation should have caused an apparent increase in availability which is contrary to our observations (table 3) that the availabilities from the addition of 2 and 3% of pork were 89 and 73% respectively.

The results of the bioassay checked well with those obtained from the determination of tryptophan in the feces. This is evident from the results presented in table 5 and table 4 where the same experimental groups were used for both types of determinations.

In calculating the unavailable tryptophan from the protein supplements it has been assumed that the tryptophan from the basal proteins is unavailable to the same extent in both the supplemented and unsupplemented diets, and, hence is proportional to food consumption. Also metabolic tryptophan

in the feces of all groups has been taken to be proportional to food consumption. That the latter assumption is true has been shown by Kuiken and Lyman ('48). Further, this assumption regarding endogenous fecal amino acids is similar to that of Mitchell ('24) and Schneider ('35) regarding endogenous fecal nitrogen.

The computation of availability by the determination of tryptophan in the feces may be subject to the criticism that the bacterial action in the lower part of the intestine may alter the distribution of amino acids in the feces. Kuiken ('52) has, however, put forward some evidence obtained by feeding sulfa drugs that this sort of interference by bacteria is insignificant.

Kuiken and Lyman ('48) have reported 100% availability of tryptophan from roast beef by assay of tryptophan in the feces with a different experimental technique and this checks with our observations. Schweigert ('48) found that the percentage availability of tryptophan for growth of chicks from raw soybean meal, fish meal and processed soybean meal was 20, 40 and 65 respectively. However, in our experiment the purified soybean proteins showed about 90% availability.

The protein mixture used in this study contained 0.06% of tryptophan and supported a growth of 7 to 8 gm per week during the two-week experimental period. The growth rate increased to about 32 to 35 gm when only 0.045% of L-tryptophan was added. Very little, if any, further improvement in growth was obtained with supplements of tryptophan higher than 0.045% which indicates that the tryptophan requirement of the weanling rat is in the neighborhood of 0.11% of the diet with this level of protein. The value of Rose and Osterling ('52) of 0.2% for the tryptophan requirement of the rat was determined using pure amino acid mixtures and included an allowance "in order to insure the presence of an adequate intake" and, therefore, may not be directly comparable. However, the value of 0.11% is also much lower than that reported by Salmon ('54) who found the tryptophan requirement with

a diet containing 20% of protein to be about 0.17% in the presence of adequate niacin.

SUMMARY

The availability of tryptophan from beef, pork, casein, egg albumin, beef fibrin, purified soybean protein and from milk powders was determined and was found to be 80 to 100% in all cases, the highest being from beef.

When the amount of supplementary tryptophan was increased either by increasing the level of the protein supplement or by adding crystalline L-tryptophan together with the protein supplement, the apparent availability was lowered.

Two ways of presenting the data and calculating availability have been discussed. The percentage availability figures arrived at with two different types of calculation did not differ significantly from each other.

The unavailable tryptophan in the feces of different experimental groups given beef, pork, egg albumin and fibrin supplements was low, correlating well with the results of the other determinations.

LITERATURE CITED

DENTON, A. E., AND C. A. ELVEHJEM 1953 Enzyme liberation of amino acids from different proteins. J. Nutrition, *49:* 221.

HANKES, L. V., W. H. RIESEN, L. M. HENDERSON AND C. A. ELVEHJEM 1948 Liberation of amino acids from raw and heated casein by acid and enzymatic hydrolysis. J. Biol. Chem., *176:* 467.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. *Ibid., 138:* 459.

KUIKEN, K. A. 1952 Availability of the essential amino acids in cottonseed meal. J. Nutrition, *46:* 13.

KUIKEN, K. A., AND M. A. LYMAN 1948 Availability of amino acids in some foods. *Ibid., 36:* 359.

KUIKEN, K. A., C. M. LYMAN AND F. HALE 1947 Factors which influence the stability of tryptophan during the hydrolysis of proteins in alkaline solution. J. Biol. Chem., *171:* 551.

MAURON, J., F. MOTTU, E. BUJARD AND R. H. EGLI 1955 Availability of lysine, methionine and tryptophan in condensed milk and milk powder. *In vitro* digestion studies. Arch. Biochem. Biophys., *59:* 433.

MITCHELL, H. H. 1924 A method for determining the biological value of protein. J. Biol. Chem., *58:* 873.

OSTERLING, M. J., AND W. C. ROSE 1952 Tryptophan requirement for growth and utilization of its optical isomers. *Ibid., 196:* 33.

RIESEN, W. H., D. R. CLANDININ, C. A. ELVEHJEM AND W. W. CRAVENS 1947 Liberation of essential amino acids from raw, properly heated and overheated soybean oil meal. *Ibid., 167:* 143.

SALMON, W. D. 1954 The tryptophan requirement of the rat as affected by niacin and level of dietary nitrogen. Arch. Biochem. Biophys., *51:* 30.

SCHNEIDER, B. H. 1935 The subdivision of the metabolic nitrogen in the feces of the rat, swine and man. J. Biol. Chem.,*109:* 249.

SCHWEIGERT, B. S. 1948 Availability of tryptophan from various products for growth of chicks. Arch. Biochem. Biophys., *19:* 265.

SCHWEIGERT, B. S., AND B. T. GUTHNECK 1953 Utilization of amino acids from foods by the rat. I. Methods of testing for lysine. J. Nutrition, *49:* 277.

———— 1954 Utilization of amino acids from foods by the rat. III. Methionine. *Ibid., 54:* 333.

THE PHYSIOLOGICAL BEHAVIOR OF CALCIUM IN THE RAT [1,2,3]

SAM L. HANSARD [4] AND H. M. CROWDER
University of Tennessee — Atomic Energy Commission Agricultural Research Program, Oak Ridge, Tennessee

(Received for publication January 24, 1957)

Numerous investigators have demonstrated that calcium absorption decreases with increasing age and progressive bone calcification (Mitchell and Curzon, '39; Hansard et al., '54a, b; Henry and Kon, '53). However, with advancing age there are increased metabolic losses of calcium and a decrease in the amount of exchangeable calcium (Hansard et al., '54b; McCay, '52) that may mask the interpretation of data from aging animals. Requirements for calcium in the rat after growth ceases have been less understood, and it is not clear whether increased age is the cause or result of either poor absorption from the tract or poor retention by the tissues or both. The need for additional information upon the physiological behavior of calcium within the animal body during the various phases of development prompted this investigation. Radioisotope procedures have made possible the separation of some of the various aspects of calcium metabolism associated with the physiological changes due to growth and

[1] This manuscript is published with the approval of the Director of the Tennessee Agricultural Experiment Station, Knoxville, Tennessee.

[2] The radioactive isotopes used in these studies were obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

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

[4] Present address: Department of Animal Industry, Louisiana State University, Baton Rouge, Louisiana.

age. These have been adapted to this study of the absorption, excretion and movement of labeled calcium in the rat during its life span.

EXPERIMENTAL

One hundred and forty-one animals selected from a highly inbred Wistar strain of rats were used in this study. Litter mates were marked, weighed and maintained on UT-AEC ration Ca-3 containing 0.4% phosphorus and 0.5% calcium

TABLE 1

Description of experimental animals used and results of concurrent calcium-radiocalcium balance studies with rats at various ages [1]

AGE OF ANIMALS	NUMBER OF ANIMALS	WEIGHT	TOTAL CALCIUM		PER CENT CALCIUM INTAKE EXCRETED [2]	PER CENT CALCIUM 45 DOSE EXCRETED [3]	
			Intake	Excretion		Oral	Intraperitoneal [4]
weeks		*gm*	*mg/day*	*mg/day*			
1.5	16	14 ± 1 [5]	..	..	..	2.10 ± 0.5	..
4.0	34	36 ± 3	28	2	7	3.0 ± 0.5	0.73 ± 0.5
12	13	176 ± 18	42	25	60	45 ± 14	3.0 ± 2
24	34	305 ± 43	65	47	72	59 ± 7	12 ± 2
48–72	27	414 ± 135	80	63	78	68 ± 12	21 ± 2
106	17	453 ± 72	85	87	102	82 ± 15	25 ± 3

[1] All animals sacrificed 96 hours after isotope administration.

[2] Includes only fecal calcium.

[3] Animals received an average dose of 20 μc of Ca^{45} and 10 to 100 μg of calcium except those animals under 30 days of age which received only one-half this dose.

[4] Total calcium values are averages for all animals; about two-thirds of animals were dosed orally and one-third intravenously with tracer levels of calcium-45 chloride.

[5] Mean ± standard deviation.

(Hansard et al., '51; Hansard and Plumlee, '54). Following definite pre-experimental periods of time on this ration, selected animals from each group were given a single oral or intraperitoneal tracer dose of calcium-45 chloride at the ages indicated in table 1. Each rat was then placed in an individual metabolism cage for 96-hour balance studies (Hansard and Comar, '53) previous to sacrifice and tissue distribution analysis. The fecal and urine samples were collected sepa-

rately, usually at 24-hour intervals. The total periodic collection and the selected tissue samples were then analyzed for calcium, phosphorus, ash and radiocalcium by methods previously described by Comar et al. ('51).

RESULTS AND DISCUSSION

Age and calcium balance. A summary of the calcium intake and excretion values for 141 rats at the different ages is given in table 1. For convenience of analysis and comparative presentation these animals were lotted into 6 age groups and the data with the mean ± the standard deviation tabulated (Snedecor, '46). It may be noted that the total percentage of fecal calcium increased markedly between 4 and 12 weeks and at a reduced rate thereafter to maturity. This adjustment of the young rat to changes in ration is rapid (Hansard et al., '51) and may account for the decreased retention of calcium after the 4-week period. The marked increase of fecal calcium in the aged animals reflects decreased absorption and increased losses from the body stores as discussed later.

Age and radiocalcium excretion. The results of the concurrent radiocalcium balance studies presented in table 1 substantiate the findings relative to decreased absorption with increasing age as shown by the total fecal calcium values. Animals receiving calcium-45 orally excreted more than those receiving a single intraperitoneal dose. Insignificant amounts of the excreted radiocalcium were observed in the urine of rats of all ages, being slightly higher following intraperitoneal administration and for the mature and aged rats.

Following oral administration of calcium-45, fecal excretion ranged from 2% in the suckling rat to 45% at 12 weeks and increased to 82% in the aged animals. Following intraperitoneal administration, however, only a small fraction of the dose was found in the feces of animals under 12 weeks of age and there was a marked increase in calcium excretion in animals after the age of sexual maturity.

When consideration was given to the rate of excretion, it was noted that, following oral administration, excretion was

low and near a uniform level throughout the 96-hour balance period for the young animals; probably representing primarily endogenous calcium losses (Hansard et al., '54a). At 4 weeks of age, however, nearly 50% of that calcium-45 excreted was in the feces at the end of 24 hours after administration. At 12 and 24 weeks of age, peak excretion was at 48 hours after dosing; and animals 48 weeks and older excreted more with about equal proportion of the dose at the 24- and 48-hour period. This was a manifestation of decreased absorption and the increased time with age required for passage of the unabsorbed calcium through the tract. A somewhat similar excretory pattern was observed following intraperitoneal administration of the isotope. In the young animal, excretion was rapid, occurring principally during the first 24 hours. With increasing age, however, peak excretion occurred during the 48-hour period after administration and the older animals ultimately excreted more of the dose. The observed increased "spill over" into the urine of older animals probably reflected the higher blood levels and a lower calcium threshold with advancing age. This time lag evidenced in the excretion of radiocalcium in older animals has been discussed in more detail by Hansard et al. ('54b) and probably represents the decreased rate of movement and mobilization, and reflects the comparative physiological state of the aging animals.

Partition of fecal calcium. Simultaneous chemical and radioisotope balance studies offer a basic means for the quantitative differentiation between that calcium passing through the gastrointestinal tract unabsorbed (exogenous) and that which reaches the feces from the body itself (endogenous). In this study, fecal endogenous calcium values were estimated by both the isotope dilution and the comparative balance procedures. The details of these methods, and their basic assumptions and significance, are discussed in the literature (Hansard, '56; Hansard et al., '54b; Hansard and Plumlee, '54). However, briefly, the *comparative balance* procedure consists of integrating the excretion data from animals dosed orally

with that of similar animals receiving a single intravenous, intramuscular, or intraperitoneal dose of labeled calcium (Hansard, '56) and employing the balance data to calculate the source and quantity of calcium. It is pointed out that this method is unique for biological availability determinations (Hansard et al., '57) in that it permits either a direct measure of absorption, or the estimation of endogenous calcium losses for use in correcting conventional balance data to give the net absorption values. In this study 4-day excretion data from animals dosed orally and that of paired rats receiving a single intravenous dose of labeled calcium permitted calculation of the percentage of dietary calcium that was absorbed (A) thus:

$$A = \frac{100 - \text{Fecal Calcium } 45}{1 - B}$$

where (B) represents that fraction of an injected dose appearing in the feces. The percentage of endogenous fecal calcium (E) was then calculated as:

$$E = A - (100 - \% \text{ Fecal Ca})$$

then:

$$\text{Egm} = \frac{E \times \text{Total Ca Intake}}{100}$$

The fact that it is also necessary to know the percentage of a single injected calcium-45 dose that will reach the feces (B) permitted adaptation for concurrent endogenous measurements by the independent *isotope dilution* procedure. This method is based upon the assumption that after injected radiocalcium reaches equilibrium, the specific activity (SA) of the endogenous (E) calcium appearing in the intestinal contents becomes equal to that of the circulating blood calcium. The extent of the dilution of endogenous calcium from the plasma then with all of the fecal calcium in the digestive tract is measured by the relative specific activities of the blood plasma (SA_p) and feces (SA_f), thereby permitting a direct calcu-

lation of the percentage contribution of each calcium source to the fecal calcium thus:

$$\frac{SA_f}{SA_r} \times 100 = E \text{ (\% fecal Ca that is endogenous)}$$

then

$$E\% \times \frac{\text{Fecal Calcium (gm)}}{100} = \text{Egm.}$$

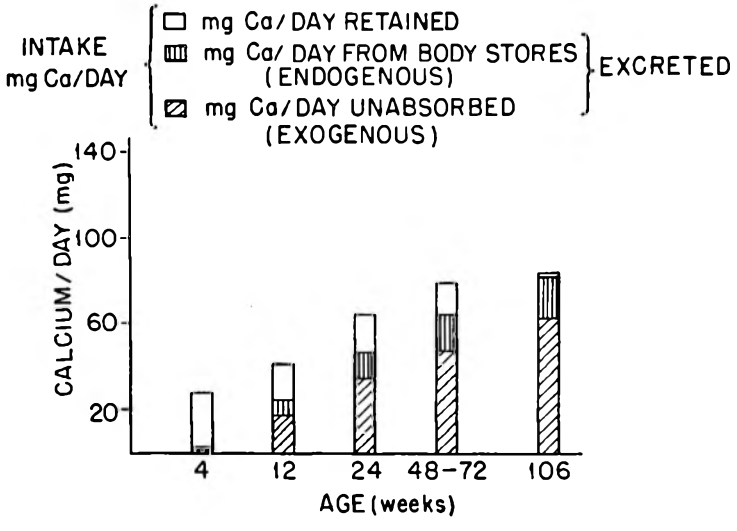

Fig. 1 The fecal endogenous and unabsorbed calcium in rats at various ages.

The results of these studies permitted the calculation of the partition values shown in table 2 and graphically summarized in figure 1. It may be observed that calcium absorption decreased from 98 to 57 to 24% in 4-, 12- and 106-week-old animals, respectively, and that the unabsorbed calcium increased from less than one in the weanling rat to 76% in the aged animal. The net calcium actually retained decreased from 97 in the weanling rat to 18% in the aged animal. There was less difference in the retained fraction (fig. 1) by animals 12 to 72 weeks of age. It is of significance that these partition values agree closely with results obtained for cattle of similar physiological age receiving normal calcium rations (Hansard et al., '54b).

TABLE 2

The partition of calcium in the gastrointestinal tract, endogenous fecal calcium, digestibility and estimated maintenance requirements of rats at various ages

AGE OF ANIMALS	AVERAGE WEIGHT	PER CENT OF Ca INTAKE			ENDOGENOUS FECAL Ca [1]		DIGESTIBILITY		REQUIREMENT FOR MAINTENANCE	
		Unabsorbed	Absorbed	Absorbed, re-excreted			Apparent [3]	True [4]	Per rat [5]	Per 100 gm
weeks	*kg*				*mg/day*	*mg/kg* [2]			*mg*	*mg*
4	0.04	2	98	0.7	1.2 ± 0.7 [6]	30	93	98	1.2	3.0
12	0.18	43	57	2	7 ± 1.8	39	40	57	12	6.6
24	0.31	54	46	6	12 ± 2.4	39	28	46	26	8.4
48–72	0.45	59	41	7	18 ± 2.0	40	22	41	44	9.8
106	0.45	76	24	6	22 ± 3.4	49	−2	24	92	20.0

[1] Calculated by the isotope dilution and comparative balance procedures (see text).

[2] Milligrams endogenous fecal calcium excreted per kilogram body weight per day.

[3] Calculated as $100 - \left(\frac{\text{Fecal calcium}}{\text{Calcium intake}} \times 100. \right)$.

[4] Calculated as $100 - \frac{(\text{Ca excreted} - \text{endog. fecal Ca.})\ 100}{\text{Ca intake}}$.

[5] Milligrams of calcium per rat day calculated as $\frac{\text{Milligrams endog. fecal Ca}}{\text{True digestibility}} \times 100.$

[6] Mean ± standard deviation.

Endogenous fecal calcium and maintenance requirements. The estimation of endogenous losses of calcium from the body permits the measurement of that calcium actually absorbed and retained by the animal and thereby makes it possible to determine experimentally the *true* digestibility of dietary calcium under normal feeding conditions. This is of considerable importance in mineral utilization and availability studies, and is basic for the interpretation of data procured from radioisotope investigations.

It is of interest to note from table 2 that daily endogenous fecal calcium increased in a regular manner with increasing weight and age (fig. 1) of the animal. Milligrams of endogenous calcium per kilogram of body weight, however, was less affected by age. Consideration of the endogenous values at 4 weeks and in the aged animal indicate a slight trend toward an increase, but after 4 weeks these values were relatively constant. The average daily values of 39 mg of endogenous calcium per kilogram of body weight for animals of all ages on this normal dietary are in agreement with values for swine (Hansard, '56) but are somewhat higher than those reported for rats by Cole et al. ('29) and are nearly three times the 16 mg per kilogram reported for cattle by Hansard et al. ('54b).

Calcium maintenance requirements for rats are essentially equal to the endogenous fecal losses, and were calculated from these and the *true* digestibility values given in table 2. For rats 12 weeks of age which had been maintained on 0.5% calcium, endogenous losses averaged 7 mg per day and the *true* digestibility values showed an average utilization of 57%, indicating a daily calcium requirement for maintenance of 12 mg of dietary calcium per day or 6.6 mg per 100 gm body weight. The calculated requirements for animals maintained on a normal calcium ration to the ages indicated are tabulated (table 2). There was a marked increase in the requirement sometimes between 4 and 12 weeks of age as shown by values of 3.0 and 6.6 mg per 100 gm body weight per day, respectively. In the old animals the high endogenous loss

and the poor absorption and decreased retention of calcium increased the daily requirements necessary to maintain this calcium status to 20 mg per 100 gm body weight. However, since the estimated requirements are based upon absorption values of a normal dietary calcium intake level this value for aged animals may be considered to have little physiological significance.

Age and tissue calcium, phosphorus and ash. The calcium content of the various tissues averaged by age groups is presented in table 3. Except for the very young animals, there were no significant effects of age upon the calcium values of the soft tissues. The high calcium tissue values demonstrated in the suckling rat may be a reflection of the physiological functions of the rapidly growing animal and of the higher equilibrium level of plasma and new bone as discussed previously by Hansard et al. ('54a). The slight increase in calcium concentration for kidney and muscle after 12 weeks of age was not significant. The bones showed the expected increase of calcium with age. The low calcium concentration in the bones of 10-day-old animals may have been due to the comparatively high moisture content, but is also indicative of the low level of calcification at which the rat starts life (Cox and Imboden, '36). Calcium content appeared to reach a maximum level in all tissues at 24 to 48 weeks of age except for the tibia epiphysis and mandible which showed a continuing increase to maturity and old age.

Phosphorus and ash were determined on all individual samples. However, the relationship to calcium at the various ages was so constant that only overall averages are presented. For all bones the ratio for calcium to phosphorus was 2.2 ± 0.3 and the ratio of calcium to ash was 0.38 ± 0.03. For all soft tissue the ratio of calcium to phosphorus was 0.038 ± 0.01, and age did not appear to significantly influence these values.

Tissue distribution of labeled calcium. A summary of calcium-45 concentration values in selected tissues of rats at various ages is presented in table 4. When tissue concentration was calculated on the basis of administered dose, the de-

TABLE 3

Calcium concentration[1] *in selected tissues of rats at various ages*[2]

TISSUE	AGE GROUPS (WEEKS)						
	1.5	4	12	24	48	72	106
Blood plasma	0.120 ± 0.025[3]	0.092 ± 0.040	0.063 ± 0.040	0.055 ± 0.012	0.066 ± 0.023	0.066 ± 0.035	0.070 ± 0.021
Gastrocnemius muscle	0.298 ± 0.030	0.091 ± 0.062	0.075 ± 0.024	0.088 ± 0.021	0.107 ± 0.051	0.094 ± 0.018	0.110 ± 0.055
Kidney	0.208 ± 0.015	0.098 ± 0.031	0.130 ± 0.04	0.132 ± 0.052	0.151 ± 0.037	0.143 ± 0.025	0.143 ± 0.10
Liver	0.249 ± 0.031	0.072 ± 0.032	0.051 ± 0.016	0.092 ± 0.032	0.093 ± 0.019	0.061 ± 0.026	0.065 ± 0.034
Spleen	0.291 ± 0.060	0.090 ± 0.021	0.089 ± 0.052	0.091 ± 0.038	0.084 ± 0.033	0.075 ± 0.031	0.084 ± 0.030
Tibia shaft	85 ± 3	111 ± 15	163 ± 24	164 ± 20	187 ± 26	207 ± 16	217 ± 16
Tibia epiphysis	70 ± 11	77 ± 20	103 ± 8	119 ± 11	142 ± 33	155 ± 30	157 ± 15
Incisors	114 ± 5	197 ± 33	208 ± 18	237 ± 8	238 ± 16	238 ± 26	233 ± 43
Molars	..	172 ± 27	208 ± 41	225 ± 35	238 ± 36	236 ± 51	245 ± 40
Mandible angle	58 ± 4	135 ± 15	159 ± 25	163 ± 16	158 ± 20	184 ± 20	164 ± 27

[1] Calcium expressed as milligrams Ca/gm fresh tissue.

[2] See table 1 for description and number of animals/age group.

[3] Mean ± standard deviation.

creased retention with age of labelled calcium was reflected in the tissue calcium-45 values. Therefore, in order to minimize effects due to absorption differences, method of administration and body weight, and to show more clearly the physiological effects of age itself upon calcium behavior all results were calculated on the basis of percentage of absorbed calcium-45 dose, corrected to a standard body weight of 100 gm (Hansard, '53). Thus the values tabulated reflect the behavior in the animal body of that radiocalcium actually

TABLE 4

Concentration[1] *of absorbed calcium-45 in selected tissues of rats at various ages*[2]

TISSUE	AGE GROUPS (WEEKS)						
	1.5	4	12	24	48	72	106
Blood plasma	0.013	0.016	0.021	0.027	0.047	0.048	0.105
Gastrocnemius muscle	0.020	0.014	0.017	0.031	0.038	0.056	0.151
Kidney	0.013	0.006	0.021	0.017	0.051	0.104	0.231
Liver	0.043	0.005	0.007	0.013	0.043	0.082	0.123
Spleen	0.019	0.010	0.021	0.026	0.067	0.079	0.123
Tibia shaft	11.2	7.9	6.6	7.5	3.7	4.1	3.5
Tibia epiphysis	12.5	9.9	12.3	25.7	20.1	22.3	21.2
Incisors	18.4	13.5	17.1	29.4	51.4	53.1	78.3
Molars	9.3	8.2	7.2	12.1	15.1	13.9	17.1
Mandible angle	12.7	9.5	10.9	14.6	10.4	9.1	7.1

[1] Concentration of Ca^{45} expressed as percentage of absorbed dose per gram fresh weight. (Calculations standardized to 100 gm body weight.)

[2] All animals sacrificed 96 hours after administration of calcium-45.

made available to it. These data confirm earlier findings with cattle (Hansard et al., '54a) indicating a definite increase in soft tissue radiocalcium concentration with age. However, as previously pointed out, the changes in total calcium concentration with age were not great. Therefore, this tissue increase must necessarily be accredited to the decreased physiological functions, particularly in the bones of the aging animals (Hansard et al., '54a). Calcium-45 concentration in bones showed less age effect but demonstrated a general increase to 24 weeks and then leveled off or decreased. This

was probably a result of both reduced exchange and contact of the bone crystals with blood calcium during the latter periods of life. In contrast, the incisor teeth showed a marked and constant increase in concentration to 106 weeks of age. This is a reflection of their continuous growth and calcification even in adult animals.

Autoradiograms of sagittal sections of the femur bones from these animals indicated the general relationship between deposition and areas of physiological functions. There was

TABLE 5

Specific activity[1] *of absorbed calcium-45 in selected tissues of rats sacrificed at ages indicated*[2]

TISSUE	AGE GROUPS (WEEKS)						
	1.5	4	12	24	48	72	106
Blood plasma	108	178	350	450	671	686	1500
Gastrocnemius muscle	66	156	213	344	345	622	1373
Kidney	62	60	161	131	340	742	1650
Liver	172	71	140	144	478	1367	1757
Spleen	66	111	233	289	838	1053	1557
Tibia shaft	131	71	41	46	20	20	16
Tibia epiphysis	178	129	119	216	142	144	135
Incisors	161	69	82	124	216	223	336
Molars	..	48	35	54	63	59	70
Mandible angle	219	70	69	90	66	49	43

[1] Specific activity expressed as per cent of absorbed Ca^{45} dose per milligram of calcium $\times 10^3$. (Calculations standardized to 100 gm body weight.)

[2] All animals sacrificed 96 hours after administration of calcium-45.

a decreased epiphyseal and periosteal deposition and an increased trabecular laydown of calcium-45 with increased age as described in detail for cattle (Hansard et al., '54a), and are not included in this report.

Age and tissue specific activity. Specific activity values for selected tissues of rats at the various ages were calculated as the percentage of absorbed dose of calcium-45 per milligram of calcium corrected to 100 gm body weight. A summary of these values is presented in table 5. It may be of particular interest to note that the specific activity for the soft tissues

increased with age of animal, and that these values were much higher for soft tissue than for bone at all ages except in the very young animals. This has been explained by Hansard et al. ('54a) in a previous study with cattle on the basic assumption that skeletal accumulation is the primary governing factor regulating ultimate availability of calcium-45 for exchange and the growth processes. Inasmuch as specific activity parallels radiocalcium concentration, values for bones showed less age effect than did the soft tissues. The high values in the very young animals were a reflection of the low calcium content and the high concentration of radiocalcium retained by the rapidly growing bones.

It has been implied that calcium-45 enters the skeleton by both exchange and the process of bone formation. Values indicate that this process is cyclic and that individual bones and areas of bone vary in degree and quantity of radiocalcium retained. Absolute values, therefore, may be masked by the concurrent resorption and accretion in the different areas and the physiological factors affecting them. Variations with increasing age indicate the influence of such biological variables as blood supply, vascularity, crystal size, hormone effects and the proportions of trabecular to cortical bone. The avidity of the young bone for the single calcium-45 dose would thus be soon masked by the accretion of stable calcium ions. The dynamic nature of calcium in the animal body is thus exemplified and values emphasize the major importance of ion exchange as the mechanism involved in the removal of calcium from blood or deposition in bone. It further facilitates interpretation of single dosage studies with radiocalcium, indicating that calcium-45 deposition in tissue gives little information as to stable calcium concentration.

SUMMARY

The excretion, absorption and movement in the body of calcium as a function of age has been studied in 141 individual rats. Consideration was given to the use of radiocalcium data

for the interpretation of the physiological behavior of the stable calcium normally present in the animal body, and the fundamental importance of balance data in radioisotope investigations was emphasized. Calcium absorption, retention and excretion rate were greatest in young animals, decreased rapidly to the age of sexual maturity and more slowly to maturity and old age. Daily endogenous fecal calcium losses and the requirements for maintenance increased slightly to maturity and markedly in the aged animal. Tissue calcium-45 concentration data, corrected for body weight and absorption differences due to age indicated that tissue deposition reflected the physiological status of the animal and its ability to handle the current calcium supply. The significance of fecal calcium partition, the estimation of endogenous calcium, true digestibility, maintenance requirements and the interpretation of radiocalcium data in terms of the behavior of stable calcium in the animal body are discussed.

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LITERATURE CITED

COLE, V. V., J. H. SPEER AND F. W. HEYL 1929 Factors influencing calcium balance. J. Am. Pharm. Assoc., *18:* 36.

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

COX, W. M., AND M. IMBODEN 1936 The mineral composition of young rats. J. Nutrition, *11:* 177.

HANSARD, S. L. 1953 Metabolism procedures and their significance in radioisotope studies with farm animals. US-AEC TID 5089: 88.

———— 1956 Tracer methods in mineral nutrition research. US-AEC TID 7512: 31.

HANSARD, S. L., AND C. L. COMAR 1953 Radioisotope procedures with laboratory animals. Nucleonics, *11:* 44.

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

HANSARD, S. L., C. L. COMAR AND G. K. DAVIS 1954a The effect of age upon the physiological behavior of calcium in cattle. Am. J. Physiol., *177:* 383.

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

———— 1951 Effects of calcium status, mass of calcium administered and age on calcium-45 metabolism in the rat. Proc. Soc. Exper. Biol. Med., *78:* 455.

HANSARD, S. L., H. M. CROWDER AND W. A. LYKE 1957 The biological availability of calcium in feeds for cattle. J. Animal Sci., *16:* 437.

HENRY, K. M., AND S. K. KON 1953 The relationships between calcium retention and body stores of calcium in the rat. Brit. J. Nutrition, *7:* 147.

MCCAY, C. M., *E. V. Cowdry's Problems of Ageing* 1952 The Williams and Wilkins Company, Baltimore.

MITCHELL, H. H., AND E. G. CURZON 1939 The Dietary Requirements of Calcium and its Significance. Acta Scientifiques et Industrelles 771. Nutr. XVIII. Herman & Cie, Paris, 101 p.

SNEDECOR, G. W. 1946 *Statistical Methods.* The Collegiate Press, Inc., Ames, Iowa.

STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF MENHADEN OIL FROM HEAT TREATMENT [1]

R. H. COMMON, E. W. CRAMPTON, FLORENCE FARMER AND A. S. W. DEFREITAS

Departments of Nutrition and Chemistry, Macdonald College, McGill University, Province of Quebec, Canada

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INTRODUCTION

Crampton et al. ('56) have compared the nutritive values of various fractions of the ethyl esters prepared from heat-polymerized linseed, soybean and sunflower seed oils. Particular attention was paid to the fraction of the distillable ethyl esters that failed to form urea adducts (the NAFD fraction). The NAFD from linseed oil was highly injurious to the well-being of young growing rats, despite the fact that the material was a bland, neutral oil. The corresponding fraction from soybean oil was injurious, though less so than linseed NAFD. The NAFD from sunflower seed oil was much less injurious than that from the other two oils. The adduct-forming fractions of the distillable esters (AFD) from all three oils were nutritionally innocuous.

Crampton et al. ('53) and Wells and Common ('53) have considered the possibility that failure of the NAFD fraction to form urea adducts might be due to formation of cyclic monomeric acids during the thermal polymerization of the triglyceride. Since that time considerable evidence has been forthcoming to demonstrate that cyclized monomeric acids are formed during heating of methyl eleostearate (Paschke and Wheeler,

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada, Journal Series no. 398.

'55) and of linseed oil (Macdonald, '56). We ourselves have applied the procedure described by Paschke and Wheeler ('55) to the NAFD fraction from linseed oil and have secured confirmatory evidence, from infrared spectrophotometry, of the presence of aromatic material in the product. Our use of the term "cyclic monomers" in our previous papers (Crampton et al., '53, '55) is justified, therefore, to the extent that this fraction has been shown to contain some cyclized material, though it is probably not composed entirely of "cyclic monomers." *Cis-trans* isomerizations may account, in part at least, for failure of NAFD fractions to form urea adducts; and comparable evidence for the presence or absence of cyclic monomeric acids in the NAFD fractions of soybean and sunflower seed oils is not yet available.

The results of comparison of NAFD fractions from linseed, soybean and sunflower seed oils, however, suggested that it could be of interest to conduct similar studies on an oil containing acids of higher unsaturation. Menhaden oil was chosen for this purpose. The results are presented in the present paper.

EXPERIMENTAL

The sample of commercial alkali-refined menhaden oil used in this work had the following characteristics: sp.gr. (25°C.), 0.9251; refractive index (25°C.), 1.4784; iodine no. (Benham and Klee, '50), 180; saponification no. 188; mean molecular weight (cryoscopic), 839; unsaponifiable, 1.04%. This oil was heated in batches of 500 gm for 15 hours at a temperature of 275°C. while passing a strong current of CO_2. The time of heating was selected on the basis of the results of preliminary kinetic studies. The heated oil was converted to the ethyl esters by transesterification in the presence of hydrochloric acid gas as catalyst (see appendix). The use of acid catalysts was preferred because of the sensitivity of the polyene acids of menhaden oil to alkali isomerization. Fractionation of the esters by urea adduct formation and distillation were performed as described by Crampton et al. ('53). In addition,

TABLE 1

Yields and characteristics of fractions of ethyl esters of menhaden oil used in feeding trial

FRACTION [1]	YIELD AS % OF TOTAL ESTERS OF HEATED OIL		REFRACTIVE INDEX 25°C.	IODINE NO.[3]	FREE FATTY ACIDS	SAP. NO.[4]	PEROXIDE VALUE [5]	
	Linseed [2] (12 hr. at 275°C.)	Menhaden (15 hr. at 275°C.)					(a)	(b)
					%		*mg/peroxide O_2/kg oil*	
AF	46 (293)[6]	45 (299)	1.4483	86	0.73	183	276	6712
NAF	54 (472)	45 (497)	1.4964	151	1.23	151	0	1047
DE	60 (294)	60 (296)	1.4517	84	1.15	186	0	861
AFD	49 (293)	34 (290)	1.4420	45	0.41	189	360	1792
NAFD	11 (300)	20 (319)	1.4659	143	1.92	169	0	1215
NDE	40 (550)	..	.....	...	...	...	...	....
WE	..	100 (314)	1.4598	156	1.32	182	0	1100

[1] AF = Adduct-forming fraction of total esters.
NAF = Non-adduct-forming fraction of total esters.
DE = "Distillable" esters.
AFD = Adduct-forming fraction of distillable esters.
NAFD = Non-adduct-forming fraction of distillable esters.
NDE = Non-distillable esters.
WE = Whole esters of unheated oil.

[2] Data for linseed oil quoted from Crampton et al. ('53).

[3] Iodine numbers determined by the method of Benham and Klee ('50).

[4] Saponification numbers determined by the method of the A.O.A.C. ('55).

[5] Peroxide values determined by the method of Skellon and Wills ('48).
(a) Value of ester fraction before its inclusion in the diet.
(b) Value determined on oil fraction extracted from the diets by cold chloroform at the conclusion of the 28-day feeding trial.

[6] Figures within parentheses are cryoscopic mean molecular weights determined in cyclohexane.

ethyl esters of the whole unheated oil were prepared by acid-catalyzed transesterification. The yields and characteristics of the various fractions are presented in table 1. It will be noted that the yield of the NAFD ester fraction was 20%, based on weight of whole esters.

The basal diet for the nutritional experiment was similar to that used in our previous experiments, except that wheat flour was replaced by ground whole wheat. The percentage compositions of the diets were as follows: — 10% level diet: whole wheat 54; dried skimmilk 19; casein 12; ester fraction 10; dried yeast 3; dicalcic phosphate 1; iodized salt 0.5; ferrous sulphate 0.1; vitamin A and D supplement 0.15; chromic oxide 0.25. Twenty per cent level diet: as above, but with 44% ground wheat and 20% ester fraction.

The experimental animals comprised 12 groups each of 10 rats. Six of these groups received each a different ester fraction at a level of 10% of the diet and the other 6 groups received the same ester fractions at a level of 20%. Ester fraction 6 (see table 1) was not included in the feeding experiment. Its place was taken by the esters of whole unheated menhaden oil which were also fed both at the 10% and the 20% level. The various diets were offered ad libitum.

The results for survival, food intake, liveweight gain, digestibility of the ether extract and gain per 1000 digested calories are presented in table 2. Tables 1 and 2 include comparable data for similar fractions from heated linseed oil. The latter data have been cited from Crampton et al. ('53).

The results presented in table 2 may be summarized in the general statement that the various fractions from heated menhaden oil had about the same effects on the well-being of the rats as had similar fractions from linseed oil in previous comparable experiments (Crampton et al., '53). A slight apparent superiority of the NAFD from menhaden oil was not sufficiently marked to warrant any assertion as to its superiority over the NAFD from linseed oil. The menhaden NAFD,

TABLE 2

Effects of esters of heated menhaden oil on survival and live weight gains of rats and the digestibility of ether extracts of the diets

FRACTION	RATS SURVIVING 28-DAY TEST		AV. LIVE WEIGHT GAINS FOR 28-DAY PERIOD		AV. FOOD INTAKE PER RAT PER 28-DAY PERIOD		APPARENT DIGESTIBILITY OF ETHER EXTRACT		AV. LIGHT WEIGHT GAIN PER 1000 CAL. DIGESTED	
	20% [1]	10%	20%	10%	20%	10%	20%	10%	20%	10%
	%	%	*gm*	*gm*	*gm*	*gm*	%	%	*gm*	*gm*
AF	100	100	118	113	238	286	94	87	105	95
	(100)[2]	(100)	(101)	(107)	(266)	(300)	(96)	(95)	(61)	(61)
NAF	0	90	...	3	...	132	..	73	..	37
	(20)	(100)	(–32)	(39)	(141)	(205)	(75)	(77)	(9)	(50)
DE	80	100	2	46	95	152	95	88	43	67
	(90)	(100)	(4)	(66)	(118)	(228)	(97)	(92)	(43)	(63)
AFD	100	100	111	122	233	261	94	91	92	103
	(100)	(100)	(57)	(108)	(193)	(299)	(96)	(96)	(54)	(62)
NAFD	10	60	–50	–13	81	96	85	87	..	..
	(0)	(0)	..	..	..	..	..	(93)	..	..
WE	100	100	61	98	165	233	93	94	73	87
	...	...	..	..	..	..	..	..	..	..
LSD (P = 0.05)			18	18	30	30			18	18
			(23)	(23)	(36)	(36)			(16)	(16)

[1] The ester fractions were fed at 20 and 10% of the diet.

[2] Figures within parentheses related to comparable data for linseed oil cited from Crampton et al. ('53).

however, was definitely and markedly inferior nutritionally to the NAFD from soybean or sunflower seed oil.

The chief interest of the present work lies in the additional evidence that it provides for the association of toxicity of the NAFD fraction of heat-polymerized oils with the presence of polyene acids in the original oil.

SUMMARY

The non-adduct-forming fraction (NAFD) of the distillable esters from heated menhaden oil was toxic to rats to a degree comparable with the toxicity of the similar fraction from heated linseed oil.

The adduct-forming fraction (AFD) of the distillable esters from the heated oil was nutritionally innocuous.

The results provide some additional evidence for an association between the toxicity of the NAFD fraction and the presence of polyene acids in the original oil.

ACKNOWLEDGMENTS

The authors are indebted to the Canadian Committee on Edible Fats and Oils, National Research Council of Canada, for financial support and for advice. They are specially indebted to Dr. C. Y. Hopkins for details of the acid-catalyzed interesterifications, and to Professor Alfred Taurins, McGill University, for determining the infrared spectrograms and advising on their interpretation. They wish also to thank Canada Packers Limited, Montreal, for donating samples of menhaden oil used in this study.

APPENDIX

A. Preparation of ethyl esters. One kilogram absolute ethanol was weighed into a tared 2-liter round bottomed flask with ground glass joint. Dry hydrochloric acid gas was passed in until the weight had increased by 20 gm.

The oil (500 gm) was then added and the mixture was refluxed for 24 hours. The mixture was then allowed to cool,

finally in a large separatory funnel. The upper layer was separated and distilled from a steam bath until the volume was reduced by one half. The concentrate was poured into 4 volumes of water, and to the mixture the lower layer from the separation was added.

The esters were extracted from the aqueous mixture with peroxide-free ethyl ether. The ether was removed under reduced pressure using a stream of N_2 instead of air. The esters thus prepared were stored under CO_2 in tightly stoppered flasks in the freezer until required.

LITERATURE CITED

A.O.A.C. 1955 Official Methods of Analysis, 8th Ed., Washington, D. C.

BENHAM, G. H., AND L. KLEE 1950 The determination of the true iodine numbers of oils containing conjugated double bond systems. J. Amer. Oil Chem. Soc., *27:* 127.

CRAMPTON, E. W., R. H. COMMON, F. A. FARMER, A. F. WELLS AND D. CRAWFORD 1953 Studies to determine the nature of the damage to the nutritive value of some vegetable oils from heat treatment. III. The segregation of toxic and non-toxic material from the esters of heat-polymerized linseed oil by distillation and by urea adduct formation. J. Nutrition, *49:* 333.

CRAMPTON, E. W., R. H. COMMON, E. T. PRITCHARD AND F. A. FARMER 1956 Studies to determine the nature of the damage to the nutritive value of some vegetable oils from heat treatment. IV. Comparison of the nutritional effects of fractions of ethyl esters of heat-polymerized linseed, soybean and sunflower seed oils as separated by distillation and urea adduct formation. Ibid., *60:* 13.

MACDONALD, J. A. 1956 Evidence for cyclic monomers in heated linseed oil. J. Amer. Oil Chem. Soc., *33:* 394.

PASCHKE, R. F., AND D. H. WHEELER 1955 Cyclization of eleostearic acid. Ibid., *32:* 473.

SKELLON, J. H., AND E. D. WILLS 1948 Iodometric methods of estimating peroxidic oxygen. Analyst, *73:* 78.

WELLS, A. F., AND R. H. COMMON 1953 Chemical aspects of thermal damage to the nutritive value of vegetable oils. II. The possible formation of cyclized or branched monomeric acyl radicals. J. Sci. Food and Agr., *4:* 233.

THE PHENYLALANINE AND TYROSINE REQUIREMENT OF THE GROWING CHICK WITH SPECIAL REFERENCE TO THE UTILIZATION OF THE D-ISOMER OF PHENYLALANINE[1]

HANS FISHER, DEWEY JOHNSON, JR. AND GILBERT A. LEVEILLE

Poultry Science Department, Rutgers University, New Brunswick, N. J.

(Received for publication January 17, 1957)

The phenylalanine requirement of the growing chick had been set at 0.9% of the diet by the National Research Council. This value represents an estimate of Almquist ('47) based on the phenylalanine content of a protein-containing diet. Grau ('47) has studied the DL-phenylalanine requirement using a free amino acid diet and reported the requirement to lie between 0.6 and 0.8%. On the basis of carcass retention, Grau concluded that the L- and D-isomers were equally active for the chick. Fisher ('56) recently studied the phenylalanine requirement of growing chicks using the L-isomer and found a requirement of 0.48% of the diet. This suggested that the D-isomer of phenylalanine was poorly utilized by chicks as had been observed for the growing rat by Armstrong ('53). The studies of Armstrong also pointed up the importance of well-balanced and adequate amino acid-containing basal rations, particularly when studying the differential utilization of the D- and L-isomers of amino acids.

The L-tyrosine requirement of the chick as set forth by the National Research Council is based on the report by Grau ('47)

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Poultry Science, New Brunswick.

that the tyrosine requirement can be satisfied by 0.6 to 0.8% of either tyrosine or phenylalanine.

With the development of a free amino acid diet which would support good growth in chicks (Fisher and Johnson, '57) in contrast to diets previously in use (Almquist and Grau, '44; Glista, '51; Lewis, '53), the present investigation was undertaken to determine directly the utilization of D-phenylalanine and thus redefine the phenylalanine and tyrosine requirements of growing chicks.

EXPERIMENTAL AND RESULTS

Day-old male chicks from a crossing of NH ♂ × Columbian ♀ were used for these studies. The chicks were reared in electrically heated battery brooders with 5 chicks per lot. Chicks were wingbanded and weighed individually at 1, 4, 7, and 10 days of age with feed consumption being recorded cumulatively for each group of 5 birds. Although the chicks were placed on the experimental ration at one day of age, evaluation of results was made on the basis of the gain made between the 4th and 10th day on experiment. During the first 4 days chicks are relatively insensitive to minor amino acid deficiencies, probably due to the presence of still unabsorbed yolk material. The basal ration used is shown in table 1.

Results of feeding various levels of D-, L-, and DL-phenylalanine are graphically depicted in figure 1. In order to obtain an estimate of the minimum L- and DL-phenylalanine requirements, a least squares method was employed (Grim-inger et al., '56). The variance about the regression line for the L-phenylalanine data was calculated when the regression included all 7 observations and when each observation for the three highest levels of L-phenylalanine was omitted from new regression lines. The regression line giving the best fit as indicated by the lowest variance is presented in figure 1. The plateau cutting the regression line was established by averaging the 4 highest observations (note that two groups of chicks are represented by the circle and dot at 0.48% L-

TABLE 1

Composition of free amino acid diet

INGREDIENTS [1]		INGREDIENTS	
	%		%
L-Arginine HCl	2.0	Corn oil	12.00
L-Glutamic acid	3.5	Mineral mix [2]	5.34
L-Histidine $HCl \cdot H_2O$	0.6	Fiber	3.00
DL-Isoleucine	2.0	Dibasic ammonium citrate	2.60
L-Leucine	1.4	Antiacid [2]	1.00
L-Lysine·HCl (95%)	1.6	Sodium bicarbonate	1.00
DL-Methionine	0.8	Vitamin mix [2]	0.15
DL-Threonine	1.2	A, D and E concentrate [2]	0.10
L-Tryptophan	0.2	Choline Cl	0.20
L-Tyrosine	0.7	Starch	to 100
DL-Valine	1.6		
Glycine	2.0		
Phenylalanine	variable		

[1] Sources of amino acids listed by Fisher and Johnson ('56).

[2] Composition given by Fisher and Johnson ('56).

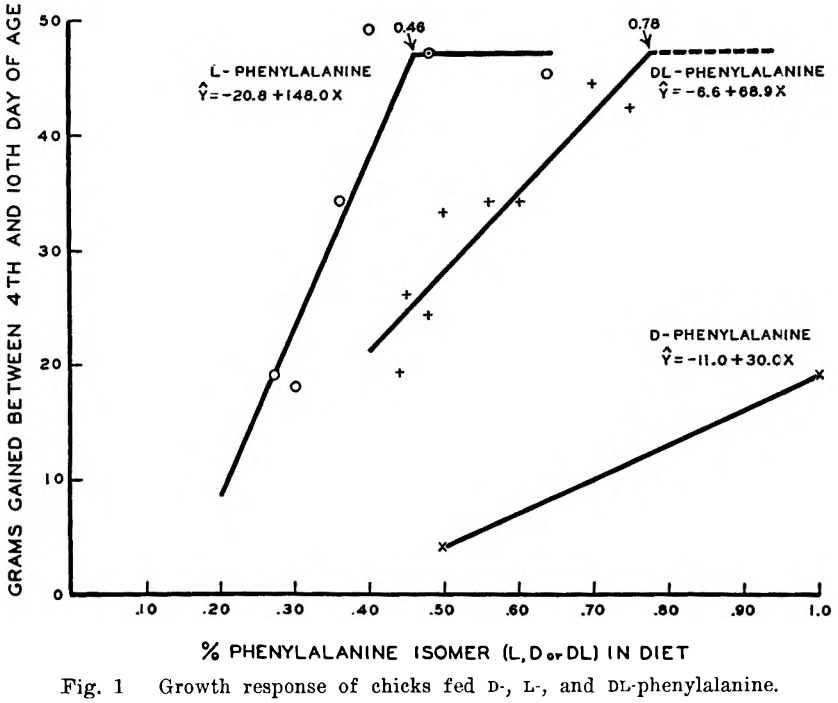

Fig. 1 Growth response of chicks fed D-, L-, and DL-phenylalanine.

phenylalanine). For DL-phenylalanine, maximum growth was not attained on the levels fed and the horizontal line (fig. 1) cutting the least squares line was therefore based on average maximum growth obtained on this diet with L-phenylalanine. The data clearly show that D-phenylalanine is not well utilized by the growing chick, either when fed as the sole source of this amino acid or when supplied in the DL-mixture.

TABLE 2

Growth response to L-*tyrosine in the presence of the minimum phenylalanine requirement and to* L-*phenylalanine in the absence of dietary tyrosine*

AMINO ACID STUDIED	GAIN 4th through 10th day	FEED UTILIZATION 4th through 10th day
%	*gm*	*gm gain/gm feed consumed*
L-Tyrosine [1]		
0.1	6 ± 3.0 [2]	0.200
0.3	35 ± 3.8	0.467
0.5	50 ± 2.0	0.543
0.7	49 ± 5.5	0.550
L-Phenylalanine [3]		
0.4	1 ± 1.9 [2]	0.040
0.6	9 ± 3.3	0.237
0.8	36 ± 1.1	0.507
1.0	46 ± 5.4	0.582

[1] In the presence of 0.46% L-phenylalanine.

[2] Standard error of the mean.

[3] In the absence of dietary tyrosine.

In the next experiment, the tyrosine requirement was determined in the presence of 0.46% L-phenylalanine, representing the minimum requirement for that amino acid as previously determined. Simultaneously, L-phenylalanine was fed at levels from 0.4 to 1.0% in the absence of dietary tyrosine. The results of these studies are given in table 2. The L-tyrosine requirement lies between 0.3 and 0.5% (calculated value, 0.4%) of the diet in the presence of the minimum requirement for phenylalanine. In the absence of dietary tyrosine, the L-phenylalanine requirement is near 1.0% although maximum

growth possibly was not quite reached at the 1.0% level in this experiment.

DISCUSSION

Since this is the first reinvestigation of an amino acid requirement for chicks using a free amino acid diet since the pioneering work of Almquist and Grau, the adequacy of the present diet deserves some attention. In the studies reported herein, the rate of gain (Grau, '47) approaches 10% as compared to maximum values of only 4% obtained on the earlier diets. This vast improvement is even more impressive when it is considered that chicks on this diet grow at approximately the same rate as on a good practical diet (Fisher and Johnson, '57).

It is important to note that the L- and DL-phenylalanine requirements of 0.46 and 0.78%, respectively, found in this investigation coincide and therefore confirm the requirements of 0.48% and 0.6 to 0.8% previously reported by Fisher ('56) and by Grau ('47). In view of the excellent agreement of these studies it is apparent that the National Research Council requirement for phenylalanine must be revised to state the requirement in terms of the L-isomer.

The poor utilization of D-phenylalanine found in the present study is in line with the report by Armstrong ('53) in the rat. The studies of Morrison et al. ('56) with D-tryptophan, showing that the poor utilization for this D-amino acid is primarily due to poor absorption offer an explanation different from that proposed by Armstrong ('53) for the poor utilization of D-phenylalanine.

Calculation [2] on the utilization of the DL-mixture relative to the L-isomer of phenylalanine shows the following. To obtain a growth response equivalent to that with 0.25, 0.35,

[2] In the equation $\hat{Y}_L = 148.0\ x_1 - 20.8 \pm 2.4$ (standard error of $\hat{y}$) and $\hat{y}_{DL} = 69.8\ x_2 - 6.6 \pm 1.3$ (fig. 1), where $\hat{y}$ = the growth response, x_1 = the per cent L-phenylalanine and x_2 = the per cent DL-phenylalanine in the diet, set $\hat{y}_L = \hat{y}_{DL}$ and solve for x_2. This gives the equation $x_2 = 2.148\,x_1 - 0.21$. By selecting values for x_1, the equivalent values for x_2 were calculated.

and 0.45% L-phenylalanine required 0.33, 0.55, and 0.76% DL-phenylalanine. The L/DL ratios were respectively 0.76, 0.64, and 0.59, indicating poor utilization of the D-isomer over the entire range of phenylalanine studied, but especially at levels permitting optimum growth.

The L-tyrosine requirement of 0.3 to 0.5% as shown herein is considerably lower than the 0.7% estimate of Grau ('47). It checks well, however, with the requirement of 0.3 to 0.4% recently reported for the rat by Armstrong ('55). The L-phenylalanine requirement in the absence of tyrosine indicates a relatively poor conversion efficiency of phenylalanine to tyrosine. This subject and its implications have been discussed in detail by Armstrong ('55).

SUMMARY AND CONCLUSIONS

The utilization of D-phenylalanine as well as the requirements for L-phenylalanine and L-tyrosine by growing chicks were the subject of this report. It was found that D-phenylalanine is very poorly utilized as indicated by slow growth obtained with the D-isomer alone as well as by a higher requirement for DL-phenylalanine than for the L form. The L-phenylalanine requirement of chicks is approximately 0.5% and the L-tyrosine requirement is not more than 0.5%. It is suggested that these values be adopted in place of the old values of 0.9% and 0.7% based on former work.

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LITERATURE CITED

ALMQUIST, H. J. 1947 Evaluation of amino acid requirements by observations on the chick. J. Nutrition, *34:* 543.

ALMQUIST, H. J, AND C. R. GRAU 1944 The amino acid requirements of the chick. Ibid., *28:* 325.

ARMSTRONG, M. D. 1953 The utilization of L-and D-phenylalanine by the rat. J. Biol. Chem., *205:* 839.

———— 1955 The phenylalanine and tyrosine requirements of the rat. Ibid., *213:* 409.

FISHER, H. 1956 The L-phenylalanine requirement of the chick. Arch. Biochem., *60:* 108.

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

———— 1957 An improved free amino acid diet for growing chicks. Poultry Sci., *36:* in press.

GLISTA, W. A. 1951 The amino acid requirements of the chick: Method and application to some of the amino acids. Doctoral Thesis, University of Illinois, Urbana.

GRAU, C. R. 1947 Interrelations of phenylalanine and tyrosine in the chick. J. Biol. Chem., *170:* 661.

GRIMINGER, P., H. M. SCOTT AND R. M. FORBES 1956 The effect of protein level on the tryptophan requirement of the growing chick. J. Nutrition, *59:* 67.

LEWIS, E. W. 1953 The relative nutritive value of whole protein and amino acid mixtures. Doctoral Thesis, University of Wisconsin, Madison.

MORRISON, W. D., T. S. HAMILTON, AND H. M. SCOTT 1956 Utilization of D-tryptophan by the chick. J. Nutrition, *60:* 47.

HISTIDINE AS AN ESSENTIAL NUTRIENT FOR THE ADULT RAT[1]

THOMAS B. MOORE[2] AND JOHN ERIC WILSON
Department of Biological Chemistry and Nutrition, School of Medicine, University of North Carolina, Chapel Hill

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The nutritional status of histidine has been the subject of some disagreement in the literature for a number of years. The conclusion of Burroughs, Burroughs and Mitchell ('40) that dietary histidine is not necessary for the maintenance of nitrogen balance in adult rats has not been supported by the results of other approaches to the question (Wolf and Corley, '39; Albanese and Frankston, '45; Frazier et al., '47; Wissler et al., '48). The bases of the differences in results have remained obscure, however, and the problem seems to merit further investigation, particularly since Bothwell and Williams ('51) have reported a similar finding in young rats. A comparison of the methods used in the various studies shows that the work of Burroughs et al. differs from the others on adult animals in one or more important points in every case. Notable differences are in the use of force-feeding to obviate any effects of nitrogen or caloric differences, and in the inclusion of all of the non-essential amino acids in the diet. The amino acids most commonly omitted in the other investigations were serine and the prolines. The first of these might conceivably condense with an imidazole moiety in an as yet unknown biosynthesis of histidine, while the prolines, like histidine,

[1] This investigation was supported in part by a grant from The Society of the Sigma Xi.

[2] Atomic Energy Commission Fellow. Present address, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

contain 5-carbon chains including heterocyclic rings. Some of these studies employed only the amino acids known to be essential for rat growth.

With these points in mind, we have studied the effects of histidine-free diets upon nitrogen balance in adult rats, and have been unable to find any evidence that this amino acid is dispensable for these animals.

METHODS AND MATERIALS

The observations were made on adult albino rats of both sexes, using diets based upon mixtures of purified amino acids (diets A and B, table 1). Cellulose was included in diet A, which was used for ad libitum feeding, in order to encourage possible intestinal synthesis of histidine or of possible unknown factors required for its biosynthesis. In diet B the cellulose was replaced by increased amounts of amino acids to facilitate force-feeding of the diet, and the maintenance of nitrogen intake during this procedure. Liver extract powder was used as a source of possible unknown factors involved in histidine biosynthesis.

Animals were kept in individual metabolism cages, and allowed free access to the diet and water at all times. Daily collections of urine, feces and any spilled food were made, and the urine and feces were analyzed separately for nitrogen by a micro-Kjeldahl method, while nitrogen intake was calculated from the amount of food consumed and a nitrogen analysis of each batch of diet. The rats were weighed daily.

Two experiments were performed. In both, the complete diet (A, table 1) was fed to let the animals become accustomed to a synthetic diet and to achieve nitrogen equilibrium. For the first set of animals (rats 1 to 5), histidine was then removed from the diet and the animals were followed for from 6 to 13 days while being fed histidine-free diet A ad libitum. Rats 1, 2, 3 and 4 were force-fed small amounts of diet toward the latter part of the experimental period.

For the second set of animals (rats 6 to 9), supplementary force-feeding was used throughout the experimental period, reckoning from the initial withdrawal of histidine. For this purpose, diet B was mixed with sufficient water to make a thin slurry and administered by stomach tube. During the initial week that the animals were on the histidine-free regimen, the amount of diet force-fed was gradually increased to test the influence of intake on nitrogen balance. Following this, the rats were returned to the complete diet for 9 days, followed by a final three-day period on the histidine-free regimen at a high level of nitrogen intake.

In both sets of experiments, each animal served as its own control.

TABLE 1

Composition of diets

CONSTITUENT	DIET A	DIET B
	gm	*gm*
Amino acid mixture [1]	135.95	186.50
Ground cellulose [2]	50.00	.....
Salt mixture [3]	40.00	40.00
Liver powder [4]	4.00	4.00
Sucrose with vitamin B_{12} and pteroylglutamic acid [5]	1.00	1.00
Sucrose with water soluble vitamins [6]	10.00	10.00
Choline chloride	2.00	2.00
Sucrose	713.55	713.00
Peanut oil with fat soluble vitamins [7]	40.00	40.00
	996.50 [8]	996.50 [8]

[1] See table 2 for composition.

[2] Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] Wesson (1932), General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Liver extract concentrate 1:20, Nutritional Biochemicals Corporation.

[5] Containing 0.1 mg of vitamin B_{12} and of pteroylglutamic acid per gram of mixture.

[6] Ten grams of mixture contains (in milligrams): niacin 28.4, calcium pantothenate 17.0, riboflavin 10.8, ascorbic acid 10.0, pyridoxine hydrochloride 6.4, thiamine chloride 5.4.

[7] Forty grams contains: 2-methyl-1,4-naphthoquinone 0.5 mg, percomorph liver oil 3 drops, cod liver oil 1 ml.

[8] The additional 3.50 gm was L(+)-histidine monohydrochloride or L(+)-glutamic acid.

TABLE 2

Amino acid mixtures

AMINO ACID	DIET A	DIET B
	gm/kg diet	*gm/kg diet*
L(+)-Arginine hydrochloride [1]	5.057	7.000
DL-Isoleucine	13.260	18.200
L(+)-Leucine	12.342	16.900
L(+)-Lysine monohydrochloride	10.000	13.700
DL-Methionine [2]	4.000	5.500
DL-Phenylalanine	7.500	10.300
DL-Threonine	8.055	11.000
DL-Tryptophan	1.600	2.200
DL-Valine	14.000	19.200
DL-Alanine	4.000	5.500
DL-Aspartic acid	7.500	10.300
L(−)-Cystine [3]	0.560	0.800
L(+)-Glutamic acid	25.410	34.800
Glycine	2.500	3.500
L(−)-Tyrosine [4]	7.000	9.600
DL-Serine	8.050	11.000
L(−)-Proline	5.120	7.000
	135.95	186.50

[1] Amino acids are from Mann Fine Chemicals, Inc., except as noted.

[2] From Dow Chemical Company.

[3] From Dr. James C. Andrews.

[4] From Eastman Kodak Company.

RESULTS

In all animals, histidine deficiency was associated with a consistently negative nitrogen balance, regardless of the intake of other amino acids or of total nitrogen. This was, as might be expected, generally accompanied by a loss of weight. Numerical data are given in table 3. Data for animal 8 are omitted because this animal developed anuria during the course of the experiment. Transitory positive nitrogen balances were shown by animals 7 and 9 when they were receiving increasingly high levels of histidine-free diet. These positive balances were one-day phenomena, and each incident was followed by a profound nitrogen loss on the succeeding day. No consistent changes in fecal nitrogen excretion were noted in any of the animals.

TABLE 3

Experiment I

Nitrogen balance and weight changes in rats fed diet A, ad libitum

(Histidine omitted from diets beginning on day 3. Animals 1, 2, 3 and 4 force-fed part of diet beginning on day 10. Nitrogen intakes and balances are given as mean daily values for the periods indicated.)

ANIMAL	SEX		DIET PERIOD (DAYS) 1–3	4–7	8–11	12–16	
1	♂	Initial wt., gm	432	423	418		
		Wt., final day, gm	431	422	406	(killed by introduc-	
		N intake, mg/day	424	298	266	tion of liquid into	
		N balance, mg/day	+12	−41	−57	lungs)	
2	♂		394	393	381	369	
			394	387	376	360	
			397	265	274	245	
			+19	−42	−38	−69	
3	♀		264	260	247	245	
			259	250	245	237	
			266	181	216	264	
			+44	−43	−21	−21	
4	♀		244	240	233	220	
			246	237	219	210	(died, day 14)
			266	175	136	112	
			+24	−26	−37	−57	
5	♀		239	244	210		
			242	219	192	185	(died, day 12)
			276	104	30		
			+15	−116	−200		

Experiment II

Nitrogen balance and weight changes in rats fed diet A, ad libitum, supplemented by force-feeding of diet B beginning day 5

(Daily intake of diet A varied from 0 to 8 gm on days 6 to 24. Nitrogen intakes and balances are given as mean daily values for the periods indicated.)

ANIMAL	SEX		DIET PERIOD (DAYS) Complete 1–3	4–5	His.-free 6–9	10–12	Complete 13–16	17–21	His.-free 22–24
6	♂	Initial wt., gm	431	434	438	408	420	422	432
		Final wt., gm	434	434	415	426	420	432	422
		N intake, mg/day	394	398	265	417	385	497	486
		N balance, mg/day	+37	+46	−88	−61	−2	+39	−5
7	♂		354	354	356	340	346	342	358
			354	358	342	349	341	352	357
			336	376	267	402	336	471	473
			−23	+8	−33	−31	−43	+30	−48
9	♂		312	308	309	289	296	297	305
			314	310	284	300	293	305	309
			637	333	224	388	352	465	473
			+32	+8	−55	−22	+3	+33	−25

DISCUSSION

There was a marked drop in the voluntary consumption of food when histidine was removed from the diets of these animals. This sort of decrease is regarded by Rose ('38) and others (e.g. Wissler et al., '48) as being almost unfailingly attributable to systemic disturbances resulting from the deficiency of an essential nutrient in this type of experiment. The decreased food intake is not regarded as the cause of nitrogen loss or as the consequence of decreased palatability of the diet. The present experiments support this view.

The results leave no doubt that histidine is an essential dietary component for the adult rat under the conditions of these experiments. The fact that animals 6, 7 and 9 gradually achieved positive nitrogen balances while being force-fed virtually their entire histidine-containing ration shows that this technique in itself does not result in a consistently negative nitrogen balance. On the other hand, these same animals lost nitrogen, even when consuming other amino acids in large amounts, on histidine-free diets.

The differences between the findings of Burroughs et al. and those of other investigators thus do not appear to be the consequences of the omission of well-established non-essential amino acids from the diets of the latter workers, nor are these differences simply the consequence of the diminished nitrogen or caloric intakes that are observed when histidine is removed from diets fed entirely ad libitum.

When the procedures of Burroughs et al. are compared with those described in this paper, a number of points of difference may be seen. For one thing, the Burroughs diets included only one vitamin, thiamine, during the experimental periods. The interrelationships between vitamins and amino acid requirements have received little attention, except for the case of niacin and tryptophan. These diets also contained norleucine, which is not generally recognized to be an ordinary component of the diet, and, indeed, has been reported by Rose ('38) to be somewhat toxic.

Another possibly significant difference is in the level of nitrogen intake. The Burroughs group maintained their animals in nitrogen equilibrium on the low intake of about 80 mg per day in contrast to the level of about 300 mg per day in the present experiments. This low level of nitrogen intake might conceivably result in a decrease in the cellular concentrations of enzymes responsible for amino acid catabolism, and this, in turn, might lead to greater efficiency in the utilization of amino acids. In such a case, the body's requirement for histidine might be met by slow synthesis in the tissues or intestine. Such an effect would be consistent with the increased requirements for amino acids in birds as the dietary protein level is increased (Almquist, '52). The present results are not conclusive on this point, but, in general, the nitrogen and weight losses were greatest at low levels of nitrogen intake. This possibility has not been definitely excluded, however, because the relatively low fat content of the present diets (4% compared to about 22% in the earlier work) made it necessary to feed greater amounts to meet caloric requirements.

Another difficulty with this line of reasoning is that Bothwell and Williams ('51) attribute their success in producing nitrogen balance in young rats on histidine-free diets to a high level of nitrogen intake. Perhaps young rats surpass adults in their ability to reutilize amino acids, as pointed out by Bothwell, Prigmore and Williams ('54) in connection with lysine deficiency. On the other hand, the results of Bothwell and Williams may not represent a true amino acid equilibrium in the tissues. Forbes and Vaughan ('54), who performed similar studies on young animals, found large amounts of undigested food in the gastrointestinal tracts of force-fed, deficient animals. That this sluggish digestion and slow movement of material through the alimentary canal may be involved in the apparent positive nitrogen balances in these experiments is also suggested by the level of fecal nitrogen excretion, which rose continuously during the period of force-feeding the deficient diet. The failure of the fecal nitrogen levels to rise in

the present experiments indicates that intestinal absorption of amino acids was unimpaired.

SUMMARY

1. Removal of histidine from the diets of adult albino rats resulted in a consistent loss of body nitrogen.

2. The inclusion of non-essential amino acids in the diet, and the maintenance of nitrogen and caloric intakes by force-feeding did not check this loss of nitrogen.

3. The differences between these results and those of Burroughs, Burroughs and Mitchell ('40) may be due to differences in dietary vitamin or fat contents, dietary amino acid compositions or to differences in levels of nitrogen intake.

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LITERATURE CITED

ALBANESE, A. A., AND J. E. FRANKSTON 1945 The dietary role of histidine in the immature and adult rat. Johns Hopkins Hospital Bull., *77:* 61–67.

ALMQUIST, H. J. 1952 Amino acid requirements of chickens and turkeys: A Review. Poultry Sci., *31:* 966–981.

BOTHWELL, J. W., AND J. N. WILLIAMS, JR. 1951 A study of histidine deficiency and nitrogen balance under ad libitum and force-feeding conditions. J. Nutrition, *45:* 245–252.

BOTHWELL, J. W., J. R. PRIGMORE AND J. N. WILLIAMS, JR. 1954 A study of the nitrogen metabolism of lysine-deficient rats. Ibid., *54:* 469–478.

BURROUGHS, E. W., H. S. BURROUGHS AND H. H. MITCHELL 1940 The amino acids required for the complete replacement of endogenous losses in the adult rat. Ibid., *19:* 363–384.

FORBES, R. M., AND L. VAUGHN 1954 Nitrogen balance of young albino rats force-fed methionine- or histidine-deficient diets. Ibid., *52:* 25–37.

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. Ibid., *33:* 65–84.

ROSE, W. C. 1938 The nutritive significance of the amino acids. Physiol. Rev., *18:* 109–136.

WESSON, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, *75:* 339–340.

WISSLER, R. W., C. H. STEFFEE, L. E. FRAZIER, R. L. WOOLRIDGE AND E. P. BENDITT 1948 Studies in amino acid utilization. III. The role of the indispensable amino acids in the maintenance of the adult albino rat. J. Nutrition, *36:* 245–262.

WOLF, P. A., AND R. C. CORLEY 1939 Significance of the amino acids for the maintenance of nitrogen balance in the adult white rat. Am. J. Physiol., *127:* 589–596.

INTERRELATED EFFECTS OF L-LYSINE AND OTHER DIETARY FACTORS ON THE GASTROINTESTINAL ABSORPTION OF CALCIUM 45 IN THE RAT AND CHICK

R. H. WASSERMAN, C. L. COMAR, J. C. SCHOOLEY [1] AND F. W. LENGEMANN [2]

Medical Division, Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tennessee [3]

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Recent studies from this laboratory have shown that certain amino acids markedly stimulated the gastrointestinal absorption of calcium 45 and strontium 89 in the rat whereas other amino acids were less effective or ineffective (Wasserman et al., '56). L-Lysine, D-lysine, and L-arginine almost doubled mineral absorption, being the most effective of the 18 amino acids examined. The favorable effect of other substances such as vitamin D, lactose, and milk, has been recognized for some time and recently emphasized by Fournier ('55) for lactose and by Lengemann et al. ('57) for milk. The mechanism of action of these substances is not definitely known although many theories have been proposed (Fournier, '55; Nicolaysen and Eeg-Larsen, '53; Maynard, '51).

In order to clarify further the favorable effect of L-lysine on Ca^{45} absorption, the metabolic interrelationship between this amino acid and other dietary factors was examined. During the course of these studies, it was also noted that

[1] Present address: Department of Physiology, University of California, Berkeley, California.

[2] Summer Participant at the Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tennessee, for 1956. Permanent address: Department of Chemistry, University of Tennessee Medical School, Memphis, Tennessee.

[3] Under contract with the United States Atomic Energy Commission.

there was a difference in the response of the rat and chick to both L-lysine and L-arginine. These and related observations are reported in this paper.

EXPERIMENTAL

In general, calcium absorption was measured by the comparative uptake in bone of a single dose of Ca^{45} ingested with and without the test substance (Wasserman et al., '56). As will be discussed later, it was sometimes necessary to determine the unabsorbed Ca^{45} by analysis of feces and intestinal contents.

For the study of the interrelationship between vitamin D and L-lysine in the rat, weanling albino males of the Carworth strain were fed the Steenbock and Black ('25) rachitogenic diet for 21 days. Three days before Ca^{45} administration, 2,000 I.U. of vitamin D [4] were given to one half of the rats; the others were given the vehicle (cottonseed oil) alone. After a 20-hour fast, the rats were given, by stomach tube, 20 microcuries of Ca^{45} and 3.6 mg of carrier calcium with or without 0.84 millimoles of L-lysine. The feces were quantitatively collected in metabolism cages and later pooled with the gastrointestinal contents at autopsy. The animals were killed at 20 hours after the Ca^{45} dosage, and the Ca^{45} in the femurs and feces was determined by methods described by Comar ('55). The response of the normal rat to either lactose or lysine or both substances was determined by similar methods.

In the corollary vitamin D study with chicks, one-week-old White Leghorn cockerels were fed the A.O.A.C. ('50) rachitogenic diet for two to three weeks. In experiment I (A, B, C,), half the birds received 50 I.U. of vitamin D [4] orally on three successive days prior to Ca^{45} administration. In experiment II, half the birds received a single oral vitamin D dose of 400 I.U. 48 hours before the Ca^{45}. The chicks not given the vitamin D received the vehicle alone (cottonseed oil). The chicks

[4] Viosterol.

were fasted for 20 hours and given, by stomach tube, about 17 microcuries of Ca^{45} and 3.6 mg of carrier calcium with or without 0.84 millimoles of the amino acid or 100 mg of skim milk solids in suspension as indicated in table 2. In group IC (table 2), the chicks received the amino acid orally (or water for the controls) and, 90 minutes later, were injected intraperitoneally with 5.0 microcuries of Ca^{45} and 0.9 mg of carrier calcium. Twenty-two hours after Ca^{45} administration, the birds were killed and their tibias were excised for radioassay.

RESULTS

L-Lysine and vitamin D each increased the absorption of ingested Ca^{45} in the vitamin D-deficient rat (table 1, experiment I), as measured by the gut absorption values or the accumulation of Ca^{45} in the femurs. When vitamin D and L-lysine were administered simultaneously, the over-all retention in bone was greater than when each substance was given singly. It appears that the rat responded additively to the amino acid and the vitamin; however, it could not be determined whether the response to the combined treatment was quantitative since almost complete absorption of Ca^{45} was attained when both substances were given. It was also observed that, in the presence of vitamin D (groups C and D), proportionally more of the absorbed Ca^{45} was retained by the femurs than when vitamin D was not administered. This re-emphasizes the multiple action of vitamin D in the rat, i.e., an effect on the absorption of calcium from the gut and another on bone mineralization or kidney excretion or both (Nicolaysen and Eeg-Larsen, '53).

In the second rat study (table 1, experiment II), lactose and L-lysine administered singly increased Ca^{45} absorption to about the same degree. It will be noted that there was an additive effect when both lactose and L-lysine were ingested together. This was similar to the response seen with L-lysine and vitamin D.

TABLE 1

Interrelationship of L-*lysine and vitamin D, and* L-*lysine and lactose, on* Ca^{45} *absorption in the rat*

EXP.	GROUP	TREATMENT	DIET	MASS OF CARRIER Ca	Ca^{45} IN FEMURS	Ca^{45} ABSORBED	PER CENT OF ABSORBED Ca^{45} IN FEMURS
				mg	*% of dose*	*% of dose*	
I	A	Control	Rachitogenic	0.9	4.9 ± 0.3 [1]	71 ± 1.7	7.0
	B	L-Lysine	Rachitogenic	0.9	6.5 ± 0.4	92 ± 2.0	7.1
	C	Vitamin D	Rachitogenic	0.9	6.4 ± 0.4	83 ± 0.7	7.8
	D	L-Lysine + vitamin D	Rachitogenic	0.9	7.5 ± 0.1	98 ± 0.3	7.7
II	A	Control	Normal	18.0	4.7 ± 0.4	...	...
	B	L-Lysine	Normal	18.0	6.1 ± 0.2	...	...
	C	Lactose	Normal	18.0	6.1 ± 0.1	...	...
	D	L-Lysine + lactose	Normal	18.0	7.3 ± 0.1	...	...

[1] Mean ± standard error of the mean. Six animals per group; mean body weight and femur ash weight of rats in experiment I were 75 gm and 122 mg, respectively; similar values for rats in experiment II were 66 gm and 141 mg, respectively.

The chicks, however, responded differently to some of these factors than did the rats. Orally administered L-lysine and L-arginine did not promote the Ca^{45} absorption from the gut of the rachitic chick (table 2, experiments IA and IB). In actuality, L-lysine inhibited Ca^{45} absorption at a high level of significance ($p < 0.01$). The favorable effect of L-arginine in the rat has been previously reported (Wasserman et al., '56). Skim milk powder also was without influence on the

TABLE 2

Effects of vitamin D, amino acids and milk on the absorption of Ca^{45} in the rachitic chick

EXP.	TREATMENT	METHOD OF Ca^{45} ADMINISTRATION	Ca^{45} IN TIBIAS	
			No vitamin D	Plus vitamin D
			% of dose	*% of dose*
IA	Control	Oral	4.5 ± 0.6 [1]	11.4 ± 0.9
	L-Lysine	Oral	2.9 ± 0.1	9.8 ± 0.4
IB	Control	Oral	4.2 ± 0.7	8.2 ± 0.6
	L-Arginine	Oral	3.9 ± 0.5	8.9 ± 0.8
IC	Control	Intraperitoneal	9.8 ± 0.8	...
	L-Lysine	Intraperitoneal	...	10.4 ± 0.2
II	Control	Oral	3.7 ± 0.3	6.9 ± 0.3
	Milk	Oral	3.6 ± 0.3	7.4 ± 0.3

[1] Mean ± standard error of the mean. Six animals per group in experiments IA, IB, IC; mean body weight and tibia ash weight of chicks in experiment I were 93 gm and 145 mg, respectively. Ten animals per group in experiment II; mean body weight and mean tibia ash weights of chicks in Experiment II = 127 gm and 184 mg, respectively.

accumulation of Ca^{45} by the tibias of the rachitic chick (table 2, experiment II). This same skim milk preparation has been shown by Lengemann, Comar and Wasserman ('57) to significantly increase Ca^{45} absorption in the young rat. Vitamin D, as expected, increased the movement of Ca^{45} from the gastrointestinal tract of the chick to the tibias.

The effect of vitamin D on bone mineralization or kidney function in the chick was less than in the rat since no differences were noted in the accumulation of intraperitoneally

injected Ca^{45} by the rachitic and the vitamin D-treated chick. This is in general agreement with the data of Migicovsky and Emslie ('50), which suggested that vitamin D does not influence calcium deposition in the skeleton of the chicks.

The influence of L-lysine, L-arginine, and skim milk powder on Ca^{45} absorption in the vitamin D-treated chicks could not be evaluated. Since the level of Ca^{45} in the tibias of the intraperitoneally injected chicks essentially represents 100% absorption, it is clear that vitamin D alone caused almost complete absorption of the isotope. Additional favorable influences of any substance were thus not observable.

DISCUSSION

When animals to be compared are under similar physiological conditions, it is generally accepted that the appearance of ingested Ca^{45} in bone is a reliable index of absorption and it was previously demonstrated that, in the rat, L-lysine affected the absorption of Ca^{45} only (Wasserman et al., '56). The data in table 1 emphasize that the action of vitamin D was slightly less effective than L-lysine in enhancing the absorption of calcium, but that the vitamin did have the additional action in raising the amount of absorbed Ca^{45} deposited in bone. This underlines the need for caution in the use of bone deposition as a measure of absorption. As shown in table 1, however, animals of the same vitamin D status responded identically to the influence of L-lysine on Ca^{45} absorption as measured by either bone deposition or fecal excretion.

In the normal and vitamin D-deficient rat, the gastrointestinal absorption of Ca^{45} was stimulated by L-lysine, L-arginine (Wasserman et al., '56), lactose, and skim milk powder (Lengemann et al., '57). The rachitic chick, however, did not respond to either L-lysine, L-arginine, or skim milk powder. Lactose, a major constituent of milk solids, would undoubtedly be without effect in the chick. There appears to be a distinct species difference in the ability of these substances to pro-

mote calcium absorption. It seems unlikely that the differences in the composition of the diets received by the experimental animals would suffice as an explanation; the rats on either a normal diet or a highly deficient diet responded to lysine whereas the chicks given a diet deficient only in vitamin D did not respond.

By indirect evidence, Lehmann and Pollack ('41–'42) suggested that amino acids would promote calcium absorption by virtue of the increased solubility of calcium in their presence. Since similar conditions, for example, pH, exist in the intestinal tract of both species (Dukes, '55), one would not expect the solubility of calcium salts as affected by amino acids or lactose *per se* to differ between species. Other physicochemical reactions, such as complex or chelate formation between the mineral and L-lysine or lactose, should also be similar in the chick and rat. Another point of similarity between these species is the response of their bacterial flora to dietary lactose. Dietary lactose leads to an increase in the number of lactic acid bacteria in both the fowl and rat (Johansson et al., '48; Nath et al., '48). Therefore, it is unlikely that physicochemical reactions or changes in the bacterial flora and their subsequent metabolism would account for the observed species differences, especially under the short duration of these experiments. The alternate hypothesis is that these dietary factors elicit their favorable response in the rat by a direct action on tissue metabolism or, more specifically, the metabolism of the intestine. However, the stimulation of the flow of digestive juices by L-lysine or lactose and the subsequent effect of these juices on calcium absorption offers another possible explanation.

The early studies of Kline et al. ('32) have shown that lactose increases calcification in the rachitic chick. This investigation was of longer duration than the presently reported experiments and could not differentiate between effects on bone mineralization *per se* or calcium absorption. The present findings that milk solids (which include lactose) have no effect on calcium absorption in the chick suggest a direct

influence of these substances on ossification. However, it has been determined in this laboratory that ingested lactose does not increase the deposition of parenterally administered Sr^{85} in the femurs of the rat. From experience in similar studies with Ca^{45} and Sr^{89} (Wasserman et al., '56), it can be inferred that lactose would also not have a direct effect on deposition of Ca^{45} in bone under the conditions of these experiments. The influence of lactose on parenteral Ca^{45} in the chick has not been investigated.

Of particular interest is the response of the rat to the combined L-lysine and vitamin D treatment. It had previously been shown that the maximum effect of L-lysine on calcium absorption occurred at a molar ratio of lysine to calcium of about 3:1 (Wasserman et al., '56). In the present experiment, the molar ratio was 9:1 and, therefore, the lysine-stimulated route was undoubtedly at saturation. Since vitamin D promoted a further response above that of L-lysine alone, it is suggested that the vitamin D and lysine do not replace each other in the calcium absorption process. Complete independence of action, however, cannot be deduced from these data. The lactose and L-lysine interaction also appeared to be additive. Again, complete independence of action cannot be eliminated in view of these limited data although it can be suggested that the two latter processes were not competitive.

SUMMARY

1. Singly administered L-lysine and vitamin D promoted Ca^{45} absorption in the vitamin D-deficient rat. The combined effect of L-lysine and vitamin D was about the sum of the effects of the individual components. L-Lysine and lactose singly increased Ca^{45} absorption in the normal rat, and their combined effect was also additive.

2. In contrast to the rat, L-lysine, L-arginine, and skim milk powder did not increase Ca^{45} absorption in the rachitic chick. Treatment of the rachitic chick with vitamin D resulted in nearly complete absorption of Ca^{45} in this species. For

this reason, the combined effect of other substances with vitamin D could not be evaluated.

3. Proportionally more of the absorbed Ca^{45} was found in the femurs of the vitamin D-supplemented rat than in the vitamin D-deficient rat. Vitamin D had no apparent effect on the deposition of absorbed Ca^{45} in the tibias of the rachitic chick.

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LITERATURE CITED

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 Official methods of analysis, 7th Ed., Washington, D. C., p. 792.

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

DUKES, H. H. 1955 *The Physiology of Domestic Animals.* Comstock Publishing Associates, Ithaca, New York.

FOURNIER, P. 1955 The metabolic action of sugars. Compte. rend., *240:* 115.

JOHANSSON, K. R, W. B. SARLES AND S. K. SHAPIRO 1948 The intestinal microflora of hens as influenced by various carbohydrates in a biotin-deficient ration. J. Bact., *56:* 619.

KLINE, O. L., J. A. KEENAN, C. A. ELVEHJEM AND E. B. HART 1932 Lactose in nutrition. J. Biol. Chem., *98:* 121.

LEHMANN, H., AND L. POLLACK 1941–1942 The influence of amino acids on transfer of phosphate in muscle extract and on the solubility of magnesium and calcium salts. J. Physiol., *100:* 17P.

LENGEMANN, F. W., C. L. COMAR AND R. H. WASSERMAN 1957 Absorption of calcium and strontium from milk and nonmilk diets. J. Nutrition, *61:* 571.

MAYNARD, L. A. 1951 *Animal Nutrition,* 3rd. Ed. McGraw-Hill Book Co., Inc., New York.

MIGICOVSKY, B. B, AND A. R. G. EMSLIE 1950 Deposition of radioactive calcium in rachitic and nonrachitic chick tibia from oral and intramuscular doses of Ca^{45}. Arch. Biochem., *28:* 324.

NATH, H., V. H. BARKI, W. B. SARLES AND C. A. ELVEHJEM 1948 Microorganisms in the cecal contents of rats fed various carbohydrates and fats. J. Bact., *56:* 783.

NICOLAYSEN, R., AND N. EEG-LARSEN 1953 The biochemistry and physiology of vitamin D. *Vitamins and Hormones, 11:* 29. Academic Press, New York.

STEENBOCK, H., AND A. J. BLACK 1925 Fat soluble vitamins. XXIII. The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. J. Biol. Chem., *64:* 263.

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

MILLET *(SETARIA ITALICA)*: ITS AMINO ACID AND NIACIN CONTENT AND SUPPLEMENTARY NUTRITIVE VALUE FOR CORN (MAIZE) [1]

AURORA S. MANGAY,* WILLIAM N. PEARSON AND WILLIAM J. DARBY

Division of Nutrition, Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee

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INTRODUCTION

"Millet" is a generic term applied to any of various small seeded cereals and forage grasses and frequently used in some countries as a general designation for sorghum, wheat or other native cereals. Botanically, distinct species are regarded as millets, the most common of which are *Panicum miliaceum* (common millet), *Setaria italica* (Italian millet), *Eleucine coracana* (finger millet or ragi), *Pennisetum typhoideum* (pearl millet) and *Echinochloa crusgalli* (Japanese barnyard millet). Anderson and Martin ('49) detail the macroscopic structure and characteristics of these various species of millet.

Millet is often considered to be a "poor man's cereal." In some regions of Eastern Europe and Africa it is consumed in the form of porridge or flatbread. In the United States it is used mainly in poultry feed. Few studies have been made of the nutritive value of this cereal. Data concerning the

* A portion of these data are taken from a thesis submitted to Vanderbilt University by one of the authors (A.S.M.) in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry.

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protein and amino acid content and nutritional value of various millets (Adolph and Cheng, '35; Swaminathan, '37; Lal, '50; and Balasubramanian et al., '52) are incomplete. The recent publication of Baptist and Perera ('56) on the essential amino acid content of 4 varieties of millet adds materially to the knowledge of these grains. The present investigation was prompted by the observation (Marie, '10; Vilter and Darby,[2]) that a lower incidence of pellagra exists in areas where this cereal is used, alone or admixed with maize, than where maize alone is eaten. This report presents data concerning the amino acid content of millet *(Setaria italica)* and observations on rats of the supplementary effect of the cereal on a maize-containing diet.

EXPERIMENTAL

The maize used was a locally grown, open pollinated variety known as "Jarvis." The millet studied was a locally grown Italian millet *(Setaria italica),* also known as German millet. Both cereals were ground to a fine meal for feeding.[3] Weanling female albino rats, 40 to 55 gm body weight, of the Sprague-Dawley strain, were randomly divided into groups of 4 to 9 animals, individually caged, and fed the indicated experimental diets. Water and food were supplied ad libitum. The rats were weighed at three-day intervals during an experimental period of 21 to 29 days.

The basic formula for the experimental diets contained 9% vitamin-free casein,[4] 5% cottonseed oil,[5] 4% salts,[6] 0.2% L-cystine and 71.8% sucrose. This was supplemented by the following quantities of vitamins in milligrams per 100 gm of diet: thiamine hydrochloride, 0.2; riboflavin, 0.3; pyridoxine, 0.25; *dl*-Calcium pantothenate, 2.0; choline chloride, 100; inositol, 10; biotin, 0.01; and pteroylglutamic acid, 0.02. Suit-

[2] Unpublished data.

[3] We are indebted to the Dixie Elevator Company for grinding the millet used in this study.

[4] Nutritional Biochemicals Corporation.

[5] Wesson Oil.

[6] Hubbell, Mendel and Wakeman ('37).

ably fortified halibut liver oil was added to the ration once each week to provide the following intakes: vitamin A, 400 I.U; vitamin D, 4 I.U.; 2-methyl-1, 4-naphthoquinone, 0.06 mg; and alpha-tocopherol, 0.7 mg. The supplements studied (maize, millet, niacin, tryptophan and lysine) were incorporated at the expense of sucrose and at the levels indicated in tables 2, 3, 4, 5 and 6.

The total nitrogen content of millet and maize was determined by the macro-Kjeldahl method (Association of Official Agricultural Chemists, '50). The estimate of protein content was made by multiplying the per cent nitrogen in a sample by 6.25. Moisture was determined by drying the sample to constant weight at 110°C.

The amino acid composition of millet and maize was determined microbiologically. Tryptophan was estimated by the procedure of Greene and Black ('44), and niacin was determined by a modification of the method of Snell and Wright ('41). Both of these assays were made at the conventional 10 ml assay level. Assays for other amino acids (lysine, tyrosine, phenylalanine, methionine, valine, leucine, isoleucine, arginine and cystine) were carried out in a total volume of 2 ml according to the procedure of Snell ('45). Commercial assay media [7] were used in the determination of all amino acids except tryptophan, threonine, and histidine. The latter amino acids were determined using *Streptococcus faecalis* 29-21 [8] and *Lactobacillus arabinosus* ATCC 8014, respectively, according to the procedure of Stokes et al. ('45).

RESULTS AND DISCUSSION

The amino acid and niacin contents of millet and maize used in the present investigations are given in table 1, along with values from the literature for whole wheat, ragi and Italian millet. The niacin content of millet found in this study (1.40 mg %) is similar to that reported by Aykroyd and

[7] Difco Laboratories, Detroit, Michigan and H and M Chemical Company, Santa Monica, California.

[8] Harrison, A. P., and P. A. Hansen, J. Bact., *59:* 197, 1950.

TABLE 1

Amino acid and niacin composition of Setaria italica, ragi, maize and wheat

(Amino acids calculated on dry basis in grams per 16 gm N.)

AMINO ACID	ITALIAN MILLET [1] (*Setaria italica*)	MAIZE	WHOLE WHEAT [2]	ITALIAN MILLET [3]	RAGI [4] (finger millet)	RAGI [5]
Arginine	2.31	8.10	4.3	...	1.20	...
Cystine	1.36	1.98	1.8	...	...	...
Histidine	1.22	2.64	2.1	...	0.30	...
Isoleucine	6.06	7.89	4.0	6.06	2.73	4.65
Leucine	10.50	9.90	7.0	10.33	4.04	8.81
Lysine	0.73	3.26	2.7	1.16	1.40	2.54
Methionine	2.42	2.64	2.5	3.00	1.32	3.18
Phenylalanine	4.22	4.04	5.1	5.62	1.30	5.09
Threonine	2.66	2.88	3.3	3.92	0.00	5.45
Tryptophan	2.02	1.19	1.2	3.00	0.66	2.41
Tyrosine	1.56	1.96	4.0	...	...	...
Valine	4.47	5.32	4.3	7.29	3.12	8.38
Niacin	1.40 mg %	2.75 mg %	5.0 mg %	...	1.40 mg %	...
Protein	12.38%	10.86%	13.2%	12.70%	3.1%	7.87%
Moisture	11.97%	11.45%	...	...	...	...

[1] Millet figures obtained by averaging results from 2 to 4 determinations.

[2] Block and Bolling ('46).

[3] Baptist and Perera ('56).

[4] Lal ('50); Balasubramanian et al. ('52).

[5] Baptist and Perera ('54).

Swaminathan ('40). However, later studies by Swaminathan ('44), indicated a much lower (0.7 mg %) niacin content of the same kind of millet and Giri and Nagana ('41) reported that Italian millet does not contain measurable amounts of this vitamin. Our analyses indicate a niacin content of millet somewhat lower than that of maize and wheat and approximately that found in ragi (finger millet). The variability in results obtained by these investigators may be due to different methods of determination. Giri and Nagana ('41) and Swaminathan ('44) used a chemical method of analysis, while Aykroyd and Swaminathan ('40) employed a microbiological method of determination.

The amino acid values obtained in this study and the results of Baptist and Perera ('56) are compared in table 1. With the exception of leucine and isoleucine, the values reported by these workers are somewhat higher than ours. Whether this discrepancy reflects actual differences in the amino acid content of the samples assayed or differences in the techniques employed cannot be stated. However, both analyses reveal a similar amino acid distribution pattern with lysine being the most limiting amino acid. The nutritional significance of the relatively high tryptophan content of Italian millet will be discussed later. In general, the assay values presented here tend to confirm the relatively high biological value of this cereal as reported by others (Niyogi et al., '34; Swaminathan, '37; Mukjerjee and Parthasarathy, '48).

A few studies of the amino acid composition of ragi (finger millet) are available and the data from the publications of Lal ('50), Balasubramanian, et al. ('52) and of Baptist and Perera ('56) are included in table 1. On the basis of the analytical data, Italian millet is clearly superior to ragi as a protein source. Except for lysine, the amino acid composition of the former is superior in every case. The amounts of tryptophan, leucine, histidine, isoleucine and phenylalanine found in Italian millet are approximately three times those

found by Lal ('50) and Balasubramanian et al. ('52) in ragi. The report of Baptist ('54) indicates a higher amino acid content of the same variety of millet. The discrepancy in results obtained by these investigators indicate the need for more reliable analyses of this cereal. However, the data of Baptist ('54) on ragi would tend to confirm the high biological value of this grain as reported by others (Niyogi et al., '34; Swaminathan, '37; and Mukjerjee and Parthasarathy, '48). It is especially important that Italian millet is quantitatively richer in total protein than is ragi. Additional confirmatory amino acid analyses for the several millets are needed to

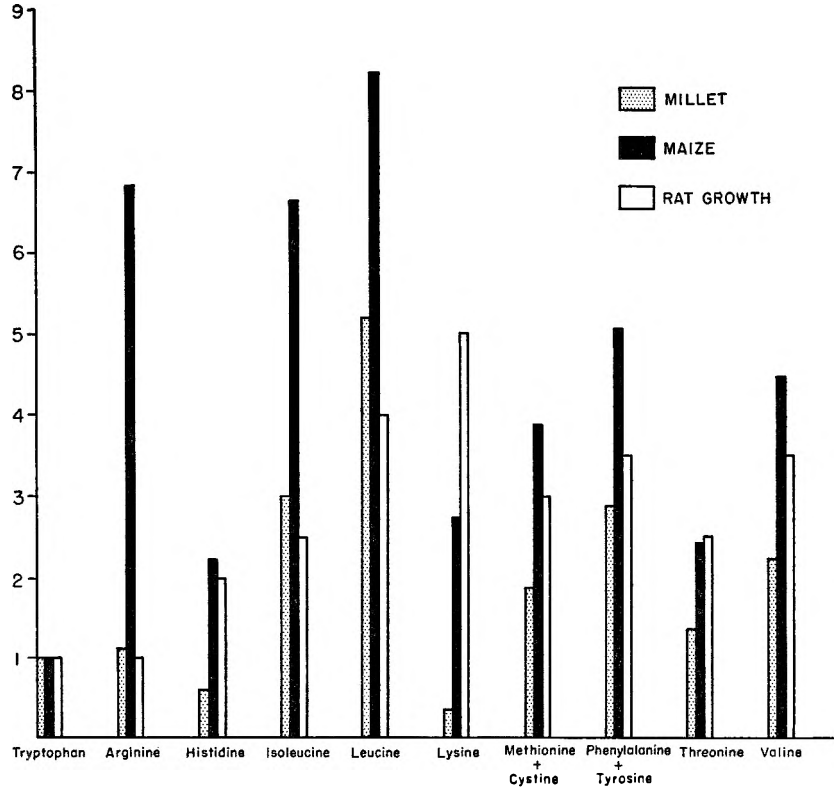

Fig. 1 Amino acid proportions in millet and maize protein compared with proportions utilized in rat growth. Amino acid proportions in protein calculated on the basis that tryptophan = 1.

permit selection of the variety of this cereal best suited for human and animal feeding.

In comparing the amino acid composition of Italian millet with that of maize, it is seen that this millet contains twice as much tryptophan as does maize, but is more limited in lysine, tyrosine, arginine and histidine. In figure 1, the proportions of amino acids of millet and maize are compared with those reported by Flodin ('53) as optimum for growth of the rat. On this basis, Italian millet appears to be most deficient in lysine, but is probably also inadequate in histidine, threonine, valine and methionine and cystine. Despite the limitations of this method of assessment, the nutritional weakness of this cereal as a sole source of protein is evident. The inadequacy as predicted from these analyses has been confirmed, and will be discussed later.

The total protein content of Italian millet is higher than that of maize or ragi, but slightly lower than wheat. The discrepancy in the protein content of ragi as reported by Lal ('50) and by Baptist ('54) requires clarification.

Data on the growth-promoting value of maize and millet are shown in table 2. These data are for rats on a basal diet containing 9% casein. All results are expressed as total weight gains and protein efficiency ratios.[9] The control animals fed the basal diet + 40% maize grew poorly, with a resulting total weight gain of but 40 gm and a protein efficiency ratio of 1.40 during the experimental period of 29 days. In this experiment, the growth was less than that obtained on the basal diet alone. Supplementing the ration with 3 mg% of niacin enhanced both the growth and protein efficiency ratios to 108 gm and 2.42, respectively. To compare the supplementary value of millet to that of maize, rats were fed the basal (9% casein) diet + 40% millet. In contrast to the maize diet, this ration satisfactorily supported growth with a total weight gain of 109 gm and a protein efficiency

[9] Protein Efficiency Ratio (P.E.R.) $= \frac{\text{grams gain in body weight}}{\text{grams protein consumed}}$.

ratio of 2.24. Increasing the content of millet to 80% did not result in an enhancement of growth beyond that attained when millet was fed at the level of 40%. In spite of the fact that these diets contained less niacin than did the comparable maize diets, it is of interest that no significant effect on the

TABLE 2

Supplementary effect of millet, maize and niacin[1] on the growth of rats receiving a basal 9% casein diet

SUPPLEMENTS TO BASAL (9% CASEIN) DIET	AVERAGE GROWTH IN 29 DAYS	P.E.R.[2]	TOTAL PROTEIN INTAKE IN 29 DAYS	NIACIN INTAKE PER 100 GM DIET	TRYPTOPHAN INTAKE PER 100 GM DIET
%	*gm*		*gm*	*mg*	*mg*
None (4)[3]	54 ± 4[4]	1.98	26.79	0.00	106
Niacin (3)	68 ± 4	2.20	30.95	3.00	106
Maize, 40 (4)	40 ± 3	1.40	28.58	1.10	133
Maize, 40 + niacin (4)	108 ± 11	2.42	44.64	4.10	133
Millet, 40 (4)	109 ± 2	2.24	48.63	0.49	192
Millet, 40 + niacin (4)	97 ± 5	2.08	46.73	3.49	192
Millet, 40 + maize, 40 (4)	117 ± 4	1.77	66.42	1.59	219
Maize, 40 + millet, 40 + niacin (4)	105 ± 4	1.63	64.46	4.59	219
Maize, 20 + millet, 40 (4)	114 ± 3	1.93	58.94	1.04	206
Maize, 20 + millet, 40 + niacin (4)	105 + 4	1.89	56.68	4.04	206
Millet, 80 (4)	111 ± 2	1.57	70.65	0.98	278
Millet, 80 + niacin (4)	112 ± 6	1.60	69.99	3.98	278

[1] All niacin supplements added were at 3 mg per 100 gm diet.

[2] Protein efficiency ratio expressed as $\frac{\text{grams gain in body weight}}{\text{grams of protein consumed}}$.

[3] Number within parentheses indicates the number of animals used in each group.

[4] Values represent average ± standard error of the mean.

growth of rats was observed when the millet-containing rations were supplemented with niacin. This is attributed to the higher tryptophan content of millet and possibly to the greater availability of its niacin. The basal diet + 40% maize supplemented with 40% millet supported growth with a resulting total weight gain of 117 gm. However, the protein

efficiency ratio was less than that found with the 40% millet diet or 40% maize + niacin diet.

The growth produced by basal diet + 40% maize + 40% millet was equivalent to that produced by the basal-maize diet + niacin. The addition of niacin to basal + maize + millet diets improved neither growth nor the protein efficiency ratio. Reducing the amount of maize to 20% in the diet did not significantly affect the growth of rats. However, the protein efficiency rations produced by diets containing 20% maize were slightly higher than those containing 40% of this cereal.

Since supplementation of the maize diets with either niacin or 40% millet enhanced both growth and the protein efficiency ratio, an experiment was devised to determine whether the supplementary effect of millet was due primarily to its niacin. The millet contained 1.40 mg% of niacin. Groups of rats were fed the basal diet + 40% maize supplemented with different levels of millet (5%, 10%, 20% and 40%) for an experimental period of 26 days. The growth of these animals was compared to that of groups receiving the basal diet supplemented with amounts of niacin (0.06, 0.12, 0.24 and 0.48 mg%) equal to that in each of the millet supplements. The results are shown in table 3. The addition of various levels of millet to basal + 40% maize diet (replacing an equivalent amount of sucrose) enhanced growth above that obtained when the equivalent amount of niacin was added. The best growth was obtained with the basal + maize diet supplemented with 20% or 40% millet. The addition of niacin alone did not enhance the growth of the rats on a 40% maize diet until the level of 0.24 mg% was reached. It should again be noted that although the growth obtained with millet supplementation was superior in every case, the protein efficiency ratios were not uniformly superior.

Inasmuch as the niacin content of millet did not appear to be the sole factor responsible for the enhancement of growth of rats receiving the basal + maize diet, a study was

made to determine whether the tryptophan content was the remaining factor involved. The tryptophan content of millet was 0.21%. Different levels of millet (5%, 10% and 20%) were added to the basal + maize diet and fed to rats for an experimental period of 27 days. The growth of these animals was compared to that of rats receiving the basal + maize diet supplemented with amounts of tryptophan (11, 22, and 44 mg%) equivalent to that contributed by millet at each level. Table 4 presents the results of this experiment. The addition

TABLE 3

Effect of varying levels of millet and its niacin equivalence on the growth of rats[1] receiving a 9% casein + 40% maize diet

SUPPLEMENTS TO 9% CASEIN + 40% MAIZE DIET	AVERAGE GROWTH IN 26 DAYS	P.E.R.	TOTAL PROTEIN INTAKE IN 26 DAYS	NIACIN INTAKE PER 100 GM DIET	TRYPTOPHAN INTAKE PER 100 GM DIET
%	*gm*		*gm*	*mg*	*mg*
None	59 ± 5[2]	1.96	30.10	1.10	133
Niacin, 0.06 mg	59 ± 4	2.35	25.11	1.16	133
Millet, 5	84 ± 11	2.44	34.41	1.16	144
Niacin, 0.12 mg	59 ± 12	2.12	27.80	1.22	133
Millet, 10	97 ± 4	2.33	41.66	1.22	155
Niacin, 0.24 mg	88 ± 11	2.75	31.97	1.34	133
Millet, 20	109 ± 2	2.16	50.38	1.34	177
Niacin, 0.48 mg	96 ± 2	3.22	29.85	1.58	133
Millet, 40	111 ± 3	1.98	56.18	1.58	219

[1] Four animals were used in each group.

[2] Standard error of the mean.

of tryptophan "equivalents" to the millet + maize diet permitted the same level of growth as that supported by the millet supplements. Furthermore, the addition of both niacin and tryptophan to the maize diet resulted in no further enhancement of growth. From these data, it is evident that the supplementary value of millet in the low-casein, raw maize diet may be attributed primarily to its tryptophan content. Again, the interesting observation is made that although growth rate is increased as the millet content of the diet is raised, the protein efficiency ratio shows a downward

drift. This suggests that amino acids, other than tryptophan, provided by the millet are used less efficiently when present in higher amounts and could be due to the fact that, as the millet content of the diet is increased, lysine becomes more and more limiting. It is unlikely that the capacity of the rat to synthesize protein has been exceeded in these experiments.

TABLE 4

Supplementary effect of millet, tryptophan and niacin-tryptophan on the growth of rats[1] *receiving a 9% casein + 40% maize diet*

SUPPLEMENTS TO 9% CASEIN + 40% MAIZE DIET	AVERAGE GROWTH IN 27 DAYS	P.E.R.	TOTAL PROTEIN INTAKE IN 27 DAYS	NIACIN INTAKE PER 100 GM DIET	TRYPTOPHAN INTAKE PER 100 GM DIET
%	*gm*		*gm*	*mg*	*mg*
None	78 ± 8[2]	2.42	32.25	1.10	133
Millet, 5	96 ± 5	2.66	38.08	1.16	144
Tryptophan, 11 mg	100 ± 3	2.81	35.59	1.10	144
Niacin, 0.06 mg + tryptophan, 11 mg	97 ± 3	2.51	38.64	1.16	144
Millet, 10	104 ± 5	2.55	40.72	1.22	155
Tryptophan, 22 mg	96 ± 1	2.64	36.35	1.10	155
Niacin, 0.12 mg + tryptophan, 22 mg	103 ± 3	2.63	39.10	1.22	155
Millet, 20	105 ± 5	2.26	46.48	1.34	177
Tryptophan, 44 mg	101 ± 6	2.67	37.76	1.10	177
Niacin, 0.24 mg + tryptophan, 44 mg	103 ± 3	2.57	40.09	1.34	177

[1] Seven animals were used in each group.

[2] Standard error of the mean.

When millet is eaten by humans it may constitute a very high percentage of the dietary. Accordingly, a further study was made of the effect of this cereal when given as the sole source of protein in the diet, i.e. without casein. The growth performance of rats fed a diet containing 89% millet for an experimental period of 21 days is given in table 5. Rats on the 89% millet diet grew poorly during the 21-day period with an average weight gain of but 18 gm and a protein efficiency ratio of 1.06. Supplementing the diet with niacin or tryptophan or both did not improve growth or the protein

efficiency ratios. However, upon addition of lysine to the 89% millet diet, growth was improved considerably with a resulting total weight gain of 83 gm and a protein efficiency ratio of 3.17. Figure 2 depicts this dramatic growth response to lysine

TABLE 5

Effect of niacin,[1] tryptophan,[2] and lysine[3] supplements on the growth of rats receiving maize, millet, and maize-millet rations

DIET	AVERAGE GROWTH IN 21 DAYS	P.E.R.	TOTAL PROTEIN INTAKE IN 21 DAYS	NIACIN INTAKE PER 100 GM DIET	TRYPTOPHAN INTAKE PER 100 GM DIET
%	*gm*		*gm*	*mg*	*mg*
Maize, 89 (4)[4]	17 ± 1[5]	1.54	11.04	2.45	60
Maize, 89 + niacin (4)	23 ± 7	1.69	13.64	5.45	60
Maize, 89 + tryptophan (4)	17 ± 3	1.36	12.36	2.45	460
Maize, 89 + niacin + tryptophan (4)	19 ± 3	1.58	12.05	5.45	460
Millet, 89 (9)	18 ± 2	1.06	16.93	1.09	191
Millet, 89 + niacin (9)	20 ± 5	1.04	19.21	4.09	191
Millet, 89 + tryptophan (9)	14 ± 1	0.80	17.55	1.09	591
Millet, 89 + niacin + tryptophan (9)	13 ± 2	0.80	16.19	4.09	591
Millet, 89 + lysine, 1 (5)	83 ± 3	3.17	26.21	1.09	191
Maize, 89 + millet, 2 (9)	21 ± 2	1.56	13.48	2.47	64
Maize, 80 + millet, 10 (9)	19 ± 3	1.44	13.19	2.32	76
Maize, 80 + millet, 5 + niacin (9)	19 ± 3	1.57	12.13	5.26	65
Maize, 80 + millet, 10 + tryptophan (9)	15 ± 1	1.38	10.86	2.32	476
Maize, 80 + millet, 10 + niacin + tryptophan (7)	13 ± 1	1.07	12.10	5.32	476
Maize, 80 + millet, 10 + niacin + lysine, 1 (6)	16 ± 1	1.54	10.42	2.32	76
Maize, 80 + millet, 10 + niacin + tryptophan + lysine, 1 (7)	52 ± 4	3.05	17.05	5.32	476

[1] Amount of niacin added was 3 mg per 100 gm of diet.

[2] Amount of tryptophan added was 400 mg per 100 gm of diet.

[3] L-Lysine was added in the form of Darvyl (du Pont Co.) containing 95% of L-lysine·HCl and 5% of D-lysine·HCl.

[4] Number within parentheses indicates the number of animals used in each group.

[5] Standard error of the mean.

supplementation. This finding confirms the amino acid analyses which indicated that this cereal was deficient in lysine.

When maize was the sole cereal and only source of protein in the diet, growth was also very poor and the addition of niacin or tryptophan or both had no effect. Addition of millet at the levels 2, 5 and 10%, as well as of niacin and tryptophan

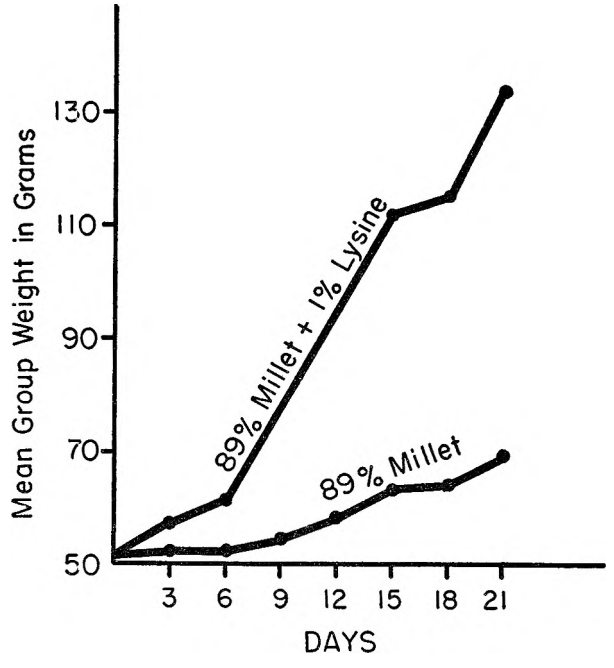

Fig. 2 Effect of lysine supplementation on the growth of rats fed an 89% millet diet.

supplements to the 80% maize diet, did not improve the growth or protein efficiency ratio. However, addition of lysine to a diet of 80% maize + 10% millet + niacin + tryptophan improved growth appreciably with a resulting total weight gain of 52 gm and protein efficiency ratio of 3.05. Lysine was without effect in the absence of niacin and tryptophan, indicating that the diet of 80% maize + 10% millet is deficient in tryptophan and lysine to about the same

degree and that the addition of both of these amino acids is necessary to obtain a good growth response.

Since lysine improved the growth of rats receiving a diet containing millet as the sole protein source, and the combination of this amino acid and tryptophan was necessary for growth on a 80% maize + 10% millet diet, a study was made to determine whether the same effect could be demonstrated on a ration containing equal parts of maize and millet. Rats were fed a diet containing 40% maize +

TABLE 6

Effect of niacin, tryptophan and lysine on the growth of rats[1] *consuming a diet containing 40% maize + 40% millet*

SUPPLEMENTS TO 40% MAIZE + 40% MILLET DIET	AVERAGE GROWTH IN 21 DAYS	P.E.R.	TOTAL PROTEIN INTAKE IN 21 DAYS	NIACIN INTAKE PER 100 GM DIET	TRYPTOPHAN INTAKE PER 100 GM DIET
%	*gm*		*gm*	*mg*	*mg*
None	14 ± 2[2]	1.10	12.73	1.59	113
Niacin, 3 mg	16 ± 1	1.21	13.17	4.59	113
Tryptophan, 400 mg	16 ± 2	1.14	14.03	1.59	513
Lysine, 1	66 ± 2	3.19	20.69	1.59	113
Niacin, 3 mg + tryptophan, 400 mg	16 ± 3	1.19	13.42	4.59	513
Niacin, 3 mg + tryptophan, 400 mg + lysine, 1	58 ± 5	3.10	18.69	4.59	513

[1] Six animals were used in each group.

[2] Standard error of the mean.

40% millet for a period of 21 days and their growth compared with that of a group receiving the same diet supplemented with combinations of lysine, niacin and tryptophan (table 6). Poor growth occurred in rats fed 40% maize + 40% millet in which the resulting total weight gain was but 14 gm and the protein efficiency ratio 1.10. The addition of lysine to the ration resulted in an increase of growth to 66 gm and a protein efficiency ratio of 3.19. Neither niacin, tryptophan, nor a combination of both, had any effect upon growth. Furthermore, these supplements did not improve the growth response to lysine.

It is evident that as the millet content of a maize diet is increased, the diet becomes more deficient in lysine and less so in tryptophan. This, combined with the fact that millet corrects a maize-induced niacin deficiency in the rat, seems to explain the low incidence of pellagra in those maize-consuming areas where millet is also used, and further suggests that under such circumstances one may encounter a limitation due to lysine. The improvement in growth of the rat brought about by the addition of lysine to a whole millet diet and to certain maize-millet mixtures is evident. Clinical manifestations of lysine deficiency have not been described in humans, and the requirements of man under such conditions is not known. The consequences of pellagra are well known. It would appear that in regions of endemic pellagra where the dietary is largely maize, one might beneficially encourage the use of millet. In such cases, careful attention should be given to selection of a variety of millet of high tryptophan content, such as the species here studied. Because of the variation in composition of millets, further knowledge of the amino acid composition of the various types is desirable. From the amino acid and niacin content of wheat, it should have a similar usefulness in maize diets and, again, the epidemiological observations are in accord with such expectations.

In regard to the nutritional value of millet in the human diet, it is of interest that Marie ('10) remarked on the absence of pellagra among the millet eaters of Egypt, and that Pieraerts ('42) observed a decreased incidence of kwashiorkor in the Belgian Congo in a year when both maize and millet were available for incorporation into the manioc bread consumed as the principal food.

SUMMARY

1. Microbiological analyses of Italian millet *(Setaria italica)* for the essential amino acids suggested that this cereal was deficient in lysine, but of high tryptophan content. The analytical data were confirmed by the findings that millet will

correct the niacin deficiency (mainly by virtue of its tryptophan content) induced in the rat by a diet of 9% casein + 40% maize and that the addition of 1% lysine to an 89% millet diet increased the growth of weanling rats 4 to 5-fold.

2. The addition of 1% lysine to a diet of 40% maize + 40% millet improved growth considerably, but no growth response was obtained by lysine addition to a diet of 80% maize + 10% millet unless niacin and tryptophan were also added. As millet is added to a maize diet the character of the diet changes from one that is deficient in niacin, tryptophan and lysine to one that is deficient in lysine alone.

3. It is concluded that the low incidence of pellagra in maize-millet-consuming areas is a reflection of the high tryptophan content of the latter cereal.

ADDENDUM

After this manuscript was submitted for publication the excellent report by Adrian and Sayerse ('54) came to our attention. These workers present microbiological data on the essential amino acid content of 4 varieties of millet cultivated in Africa and India. In contrast to the millet which we have studied, these varieties were found to be relatively rich in lysine and more limiting in tryptophan. Otherwise, the cereals were comparable.

LITERATURE CITED

ADOLPH, W. H., AND F. W. CHENG 1935 The biological value of mixed cereal proteins. Chem. J. Physiol., *9:* 245.

ADRIAN, J., AND C. SAYERSE 1954 Les Plantes Alimentaires de l'Quest Africain. II. Composition des Mils et Sorghos du Senegal. Gouvernement General da l'Afrique Occidentale Francaise (Dakar).

ANDERSON, E., AND J. MARTIN 1949 World production and consumption of millet and sorghum. Econ. Bot., *3:* 265.

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS 1950 Official and Tentative Methods of Analyses. Seventh Edition.

AYKROYD, W. R., AND M. SWAMINATHAN 1940 The nicotinic acid content of cereals and pellagra. Ind. J. Med. Res., *27:* 667.

BALASUBRAMANIAN, S. C., M. RAMACHANDRAN, T. JISWANATHA AND S. S. DE 1952 Amino acid composition of Indian foods. Part I. Tryptophan, leucine, isoleucine and valine content of some cereals. Ind. J. Med. Res., *40:* 73.

BAPTIST, N. G. 1954 Essential amino acids of some common tropical legumes and cereals. Brit. J. Nutrition, *8:* 218.

BAPTIST, N. G., AND B. P. M. PERERA 1956 Essential amino acids of some tropical millets. Ibid., *10:* 334.

BLOCK, R. J., AND D. BOLLING 1951 The amino acid composition of proteins and foods. Second Edition. Charles C Thomas, Springfield, Illinois.

FLODIN, N. W. 1953 Amino Acids and Proteins. Their Place in Human Nutrition Problems. J. Agri. Food Chem., *1:* 222.

GIRI, K. V., AND B. NAGANNA 1941 An adsorption method for the estimation of nicotinic acid content of foodstuffs. Ind. J. Med. Res., *29:* 125.

GREENE, R. D., AND A. BLACK 1944 Microbiological assay of tryptophane in proteins and foods. J. Biol. Chem., *155:* 1.

HARRISON, A. P., AND P. A. HANSEN 1950 Lactobacilli from turkeys. J. Bact., *60:* 543.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 New salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

LAL, S. B. 1950 Microbiological assay of amino acids in gram and ragi. Ind. J. Med. Res., *38:* 131.

MARIE, A. 1910 (Translated by C. H. Lavinder and J. W. Babcock) Pellagra. The State Company Publications, Columbia, South Carolina, pp. 434.

MUKJERJEE, R., AND D. PARTHASARATHY 1948 Studies of the biological value of the proteins of certain poultry feeds. Ind. J. Vet. Sc., *18:* 51.

NIYOGI, S. P., N. NARAYANA AND B. G. DESAI 1934 The nutritive value of Indian vegetable foodstuff. Part V. The nutritive value of ragi *(Eleusine coracana).* Ind. J. Med. Res., *22:* 2.

PIERAERTS, G. 1942 Kwashiorkor in Africa. Rec. Trav. Sc. Med. (Congo Belge), *1:* 104.

SNELL, E. E. 1945 Microbiological assay of amino acids. Adv. Prot. Chem., *2:* 118.

SNELL, E. E., AND L. D. WRIGHT 1941 A microbiological method for the determination of nicotinic acid. J. Biol. Chem., *139:* 675.

STOKES, J. L., M. GUNNESS, M. DWYER AND M. CASWELL 1945 Microbiological methods for the determination of amino acids. Ibid., *160:* 35.

SWAMINATHAN, M. 1937 The relative value of the protein of certain foodstuff in nutrition. Ind. J. Med. Res., *25:* 381.

———— 1944 Nicotinic acid of Indian foodstuff. Ibid., *32:* 39.

TERATOGENIC EFFECTS OF PANTOTHENIC ACID DEFICIENCY IN THE RAT

MARJORIE M. NELSON, HOWARD V. WRIGHT, CATHERINE D. C. BAIRD AND HERBERT M. EVANS[1]

Institute of Experimental Biology, University of California, Berkeley

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Nelson and Evans ('46) demonstrated that pantothenic acid deficiency in the rat resulted in fetal death with resorption or in undersized young, some of which were stillborn. Since then, Lefebvres-Boisselot ('51) has observed a high incidence of cerebral and eye defects, digital hemorrhages and edema in rat fetuses from pantothenic acid-deficient mothers. Zunin and Borrone ('54) have reported the occurrence of similar anomalies in the offspring of pregnant rats given pantothenic acid-deficient diets containing the antimetabolite, pantoyltaurine. These reports have prompted our reinvestigation of the effects of pantothenic acid deficiency on embryonic development in the rat. The effects of the vitamin antimetabolite, omega-methyl-pantothenic acid, have also been studied.

METHODS

Stock female rats of the Long-Evans strain, 60 to 65 days of age, were placed on the pantothenic acid-deficient diet on the day of breeding; others were bred after approximately 4, 10 or 20 days of the vitamin deficiency and continued on the deficient diet. Control animals received the pantothenic acid-deficient diet for 18 to 20 days before breeding and

[1] Presented in part at the 20th annual meeting of the American Institute of Nutrition, April, 1956 (Evans et al., '56). This research was aided by grants from the United States Public Health Service A-841 and the Roche Anniversary Foundation.

then were given the vitamin-supplemented diet throughout gestation. Twenty to 24 rats were used for each experimental group.

To determine the effects of the vitamin deficiency when limited to the first half of pregnancy, rats of the same age were placed on the pantothenic acid-deficient diet for the first 12 or 14 days of gestation and then given the vitamin-supplemented diet for the remainder of the gestation period. To test the accentuating effects of the antimetabolite, other rats were maintained on the vitamin-deficient diet for the first 9 or 11 days and then given the deficient diet containing the antimetabolite [2] for only two or three days. Following the antimetabolite period, the animals received the vitamin-supplemented diet for the remainder of gestation.

All rats were autopsied on the 21st day of gestation, one day before parturition; the fetuses were removed by Cesarean section and examined macroscopically for abnormalities.

The pantothenic acid-deficient diet was composed of 18% alcohol-extracted casein, 70% sucrose, 8% hydrogenated vegetable oil [3] and 4% salts [4]. Crystalline vitamins per kilogram of diet were: 300 μg of *d*-biotin, 5 mg of 2-methyl-1, 4-naphthoquinone, 5 mg of thiamine HCL, 5 mg of pyridoxine HCL, 5.5 mg of pteroylglutamic acid, 10 mg of riboflavin, 10 mg of *p*-aminobenzoic acid, 20 mg of niacin, 400 mg of inositol, and 1 gm of choline chloride. The control diet contained 50 mg of *d*-calcium pantothenate per kilogram of diet. All rats received weekly a fat-soluble vitamin mixture containing 800 U. S. P. units of vitamin A, 115 chick units of vitamin D, 6 mg synthetic *dl*-alpha-tocopherol, and 650 mg corn oil [5].

With respect to vitamin B_{12}, it seems to be well established that supplements of this vitamin are unnecessary for short-

[2] Five to 10 gm of sodium omega-methyl pantothenate were used per kilogram of diet. The compound was obtained from the California Foundation for Biochemical Research, Los Angeles, California.

[3] Crisco or Primex.

[4] Salts 4 of Hegsted et al. ('41).

[5] Mazola.

term experiments in the rat unless specially purified proteins are used or acute stress conditions imposed. Stock rats of the Long-Evans strain have been shown to have sufficient stores of vitamin B_{12} to meet the stress of lactation on purified diets [6]. In studies on riboflavin deficiency (Nelson et al., '56), supplements of vitamin B_{12} were given but it was not possible to detect any significant differences related to this vitamin.

TABLE 1

Effect of pantothenic acid deficiency on fetal development in the rat

DEFICIENCY PRIOR TO BREEDING	RATS BRED	WT. CHANGE DURING GESTATION	RESORPTIONS	LITTERS	YOUNG		
					Total no.	Av. no. per litter	Abnormal
days	*no.*	*gm*	*%*	*%*			*%*
Pantothenic acid-deficient diets throughout gestation							
0	23	+ 74	22	78	160	8.9	14
4	20	+ 53	32	68	87	6.7	29
8	21	+ 27	57	43	45	5.0	20
10	20	+ 29	70	30	44	6.3	16
20	21	— 6	100	0	0		
Pantothenic acid-supplemented diets throughout gestation							
19	24	+115	0	100	249	10.4	0

RESULTS

Table 1 shows that when pantothenic acid deficiency was instituted at the beginning of pregnancy, fetal death with resorption of the entire litter occurred in 22% of the animals. In the living young, 14% showed macroscopic abnormalities. When the deficiency was started just 4 days before breeding, embryonic development was more severely affected, the incidence of abnormal young increasing to 29% and fetal death with resorption to 32%. With longer periods of deficiency before breeding, fetal death with resorption of the entire litter increased, and when the deficiency was instituted 20 days before breeding all animals resorbed. The pregnant

[6] Unpublished data.

rats in all groups exhibited no external signs of the vitamin deficiency, e. g. greying of the fur, dermatitis, porphyrin deposition, but did not gain as much weight during the gestation period as did vitamin-supplemented controls. However, it may be noted that even one month of the vitamin-deficient diet (10 days before breeding plus 21 days of pregnancy) did not result in a loss of body weight. Addition of pantothenic acid to the deficient diet throughout the entire gestation period

TABLE 2

Effect on fetal development of pantothenic acid deficiency accentuated by an antimetabolite for two or three days

DEFICIENT DIET	ANTIMETABOLITE PERIOD [1]	RATS BRED	WT. CHANGE DURING GESTATION	RESORPTIONS	LITTERS	YOUNG		
						Total no.	Av. no. per litter	Abnormal
days	*days*	*no.*	*gm*	*%*	*%*			*%*
0–12	none	10	+116	0	100	94	9.4	0
0–11	9–11 9–12	11	+ 98	27	73	71	8.9	15
0–14	none	12	+107	0	100	101	8.4	10
0–13	10–13 11–14	8	+ 94	25	75	54	9.0	24

[1] The pantothenic acid-deficient diet contained 5 to 10 gm per kilogram of the antimetabolite, omega-methyl-pantothenic acid during this period; prior to this the deficient diet without the antimetabolite was given.

prevented effects from the 18 to 20 days of deficiency before breeding and fetal development was normal.

Table 2 shows that fetal development was only slightly disturbed when the pantothenic acid-deficient diet was given during the first 12 or 14 days of pregnancy. All animals had living young at autopsy and only a few abnormal young (0 to 10%) were found. If the deficiency was accentuated by the addition of the antimetabolite, omega-methyl-pantothenic acid, to the diet for only two or three days during the second week of pregnancy, embryonic development was seriously disturbed. Fetal death with resorption occurred in one fourth

(25 to 27%) of the pregnancies and 15 to 24% of the living young were abnormal. Accentuation of pantothenic acid-deficiency in the rat by this antimetabolite is in agreement with the findings of other investigators, e. g. Shinazi et al. ('50).

The anomalies observed macroscopically in 102 abnormal offspring (table 3) included those defects previously reported by other investigators, namely, exencephaly, (figs. 1 and 2), hydrocephalus, anophthalmia, microphthalmia, digital hemorrhages (figs. 7 to 10) and edema. In addition, interventricular septal defects, anomalies of the aortic arch pattern,

TABLE 3

Incidence and types of macroscopic abnormalities resulting from pantothenic acid deficiency in 102 abnormal 21-day fetuses

	%
[1] Microphthalmia and anophthalmia	32
[1] Edema	31
Hydronephrosis and hydroureter	31
Clubfoot, tail defects and cleft palate	23
[1] Digital hemorrhages	19
[1] Exencephaly and hydrocephalus	13
Dermal defects	13
Cardiovascular anomalies	12

[1] Anomalies reported by other investigators.

hydronephrosis and hydroureter (figs. 5 and 6), clubfoot, tail defects, cleft palate, and dermal defects (figs. 3 and 4) were observed. Single examples of undescended testes and apparent absence of the kidneys were also found. These abnormalities were similar to those observed in studies on other teratogenic dietary deficiencies, e. g. pteroylglutamic acid and riboflavin (Nelson et al., '52, '55, '56) with the exception of the digital hemorrhages and dermal defects. According to Giroud et al. ('55), the digital hemorrhages (figs. 7 and 9) are followed by degeneration of skeletal elements and sometimes by "spontaneous amputation" of the digits so that malformed paws or ectrodactyly resulted (figs. 8 and 10).

The dermal defects (figs. 3 and 4) consisted of marked hypoplasia of epidermis and dermis over small or large areas in the occipital region and in the region overlying the vertebral column; the stratum corneum and hair follicles were absent in these areas.

DISCUSSION

The data presented demonstrate the multiple teratogenic effects of pantothenic acid deficiency in the rat. The anomalies observed in this study were not limited to the nervous system and subcutaneous tissue as reported by other investigators, but included defects of the cardiovascular, urogenital and skeletal systems together with defects of the body wall. The majority of anomalies were similar to those reported for other teratogenic vitamin deficiencies although fewer types, particularly those of the skeleton, were observed. The incidence of abnormal young (10 to 29%) reported in this study is similar to that found by Zunin and Borrone ('54) but considerably lower than that reported earlier by Lefebvres-Boisselot ('51) for a small number of pregnancies, i. e. 95%. Lefebvres-Boisselot removed the fetuses during the latter half of pregnancy from the 13th to the 20th days, thus obtaining many severely affected fetuses that would probably not have survived to the 21st day of gestation, the day of autopsy for this study.

This study illustrates the relatively slight effect of the deficiency upon the maternal organism in contrast to the severe effects upon the developing embryo. This characteristic of teratogenic dietary deficiencies has been emphasized by many investigators, e. g. Giroud ('54), Warkany ('55), and Nelson et al. ('55). No external signs of pantothenic acid deficiency could be observed in the pregnant rats which gained 50 to 75 gm during gestation while one-third to two-thirds of the embryos were undergoing abnormal development or death with resorption. The pantothenic acid requirement for developing embryos with placentas on the 14th day of gesta-

tion has been estimated by Lefebvres-Boisselot ('55) to be approximately 9 mg per kilogram (weight of conceptus) in contrast to reported requirements of 3 to 4 mg per kilogram body weight at birth, 1 to 2 mg per kilogram at weaning, and 0.5 mg per kilogram at 10 weeks of age.

The deleterious effects of a transitory deficiency of pantothenic acid induced by the antimetabolite during the second week of pregnancy are similar to those found for a transitory deficiency of either pteroylglutamic acid or riboflavin during this period. Fetal damage produced during the critical period of embryonic differentiation and organogenesis cannot be reversed by vitamin supplementation later in pregnancy. The results demonstrate the necessity of pantothenic acid for early embryonic development in the rat.

SUMMARY

Multiple congenital anomalies in rat fetuses resulted when pantothenic acid deficiency was instituted on the first day of gestation or 4 to 10 days before breeding and continued throughout pregnancy. When the vitamin deficiency was limited to the first 12 or 14 days of the gestation period, few anomalies were observed. However, addition of the antimetabolite, omega-methyl- pantothenic acid, to the deficient diet for the last two or three days of this period accentuated the deficiency and fetal death with resorption of the entire litter resulted in some cases; in other cases more abnormal young were found than in the absence of the antimetabolite.

The abnormalities observed included those previously reported, namely, cerebral and eye defects, digital hemorrhages and edema and, in addition, interventricular septal defects, anomalies of the aortic arch pattern, hydronephrosis and hydroureter, clubfoot, tail defects, cleft palate and dermal defects.

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LITERATURE CITED

EVANS, H. M., C. D. C. BAIRD AND H. V. WRIGHT 1956 Multiple congenital abnormalities resulting from pantothenic acid deficiency in the rat. Fed. Proc., *15:* 549.

GIROUD, A. 1954 Malformations embryonnaires d'origine carentielle. Biol. Reviews, *29:* 220.

GIROUD, A., J. LEFEBVRES, H. PROST AND H. DUPUIS 1955 Malformations des membrés dues à des lésions vasculaires chez le foetus de rat déficient en acide pantothenique. J. Embryology and Exper. Morphology, *3:* 1.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of the chick. J. Biol. Chem., *138:* 459.

LEFEBVRES-BOISSELOT, J. 1951 Role tératogene de la déficience en acide pantothénique chez le rat. Ann. Med. (Paris), *52:* 225.

———— 1955 Contribution à l'étude des besoins en acide pantothénique de l'embryon et du foetus. Résultats expérimentaux obtenus chez le rat. Archiv. des Sciences Physiologiques, *9:* 145.

NELSON, M. M., AND H. M. EVANS 1946 Pantothenic acid deficiency and reproduction in the rat. J. Nutrition, *31:* 497.

NELSON, M. M., C. W. ASLING AND H. M. EVANS 1952 Production of multiple congenital abnormalities in young by maternal pteroylglutamic acid deficiency during gestation. Ibid. *48:* 61.

NELSON, M. M., H. V. WRIGHT, C. W. ASLING AND H. M. EVANS 1955 Multiple congenital abnormalities resulting from transitory deficiency of pteroylglutamic acid during gestation in the rat. Ibid. *56:* 349.

NELSON, M. M., H. V. WRIGHT, C. W. ASLING AND H. M. EVANS 1955 Multiple congenital abnormalities in the rat resulting from riboflavin deficiency induced by the antimetabolite galactoflavin. Ibid. *58:* 125.

SCHINAZI, L. A., W. DRELL, G. H. BALL AND M. S. DUNN 1950 Toxicity and effect of ω-methylpantothenic acid on blood-induced infections of plasmodium lophurae in the chick. Proc. Soc. Exp. Biol. Med., *75:* 229.

WARKANY, J. 1955 Congenital malformations induced by maternal dietary deficiency. Nutrition Reviews, *13:* 289.

ZUNIN, C., AND C. BORRONE 1954 Embriopatie da carenza di acido pantotenico; effetto della, pantoiltaurina, antivitamina dell'acido pantotenico. Acta Vitaminologica, *8:* 263.

PLATE

PLATE 1

EXPLANATION OF FIGURES

Lateral views of the head in 21-day fetuses from mothers maintained on the pantothenic acid-deficient or supplemented (control) diet, × 3.4

1 Control.

2 Pantothenic acid-deficient: exencephaly.

Dorsal views of 21-day fetuses, × 2.25

3 Control.

4 Pantothenic acid-deficient: marked hypoplasia of epidermis and dermis over an extensive area from the region between the scapulae almost to the lower lumbar region; the stratum corneum and hair follicles are absent in this area.

Dissections of 21-day fetuses showing the urogenital system, × 3.0

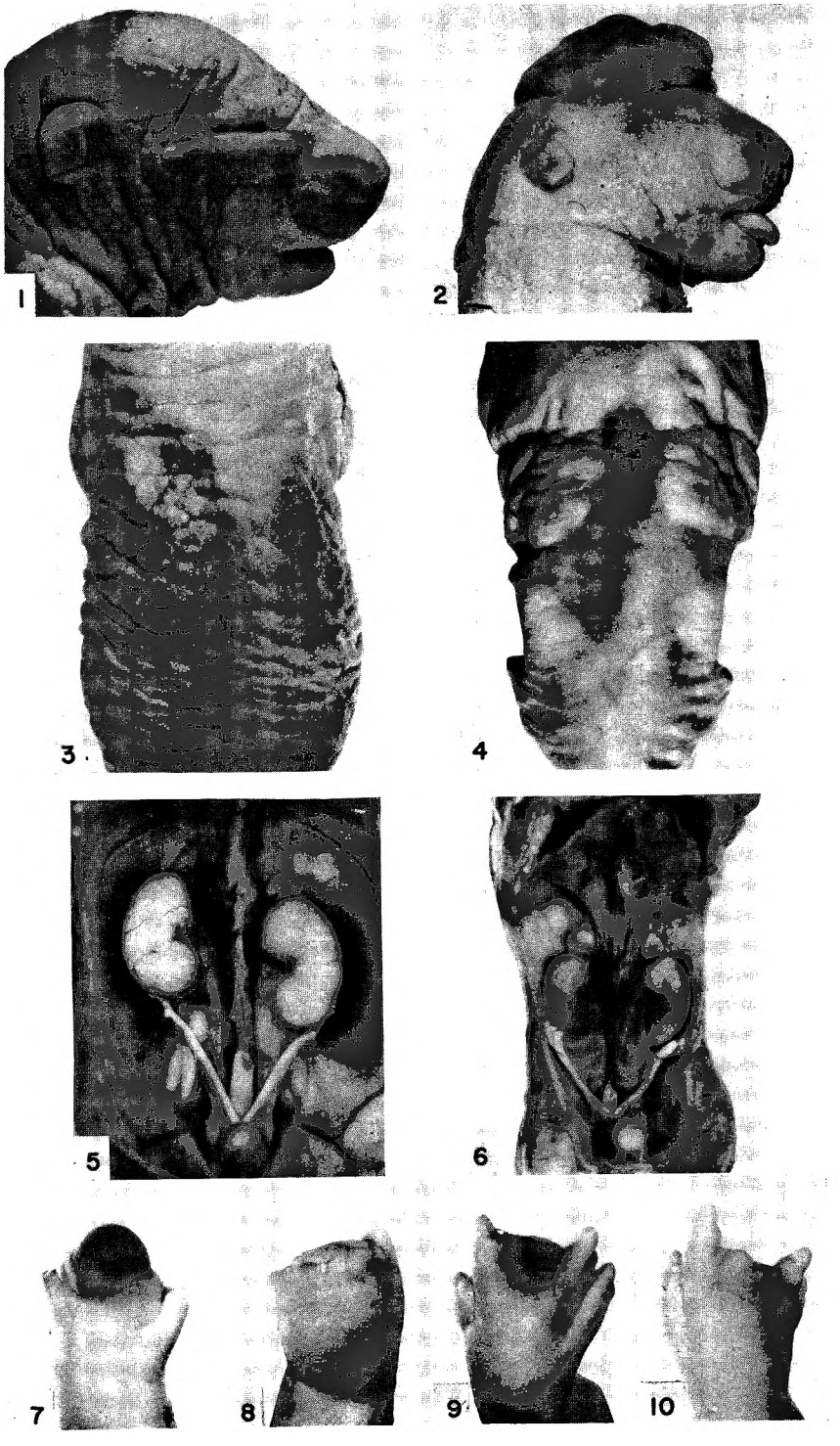
5 Control, showing kidneys, ureters, bladder and uteri.

6 Pantothenic acid-deficient: hydronephrosis and hydroureter.

Dorsal views of paws from 21-day fetuses from mothers maintained on the pantothenic acid-deficient diet, × 9.0

7,9 Digital hemorrhages.

8,10 Malformed paws showing apparent "amputation" of the digits following hemorrhage and necrosis.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THE PYRIDOXINE REQUIREMENT OF THE BABY PIG [1,2]

E. R. MILLER, D. A. SCHMIDT, J. A. HOEFER AND R. W. LUECKE

Department of Animal Husbandry, Animal Pathology and Agricultural Chemistry, Michigan State University, East Lansing

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Chick et al. ('38), Hughes and Squibb ('42), Wintrobe et al. ('43), Lehrer et al. ('51) and Moustgaard ('53) have described the various clinical and subclinical symptoms which compose a syndrome of pyridoxine deficiency in the suckling and the weanling pig. The work of Wintrobe et al. ('43) and of Moustgaard et al. ('52) indicated that 40 μg of pyridoxine per kilogram of body weight daily is sufficient to prevent the occurrence of these symptoms in the weanling pig. However, Moustgaard ('53) found it necessary to supply twice this amount to produce maximum rates of body weight gain and protein utilization.

The present series of trials was designed to determine the pyridoxine requirement of the baby pig on a synthetic-milk diet. The purpose of this paper is to submit data which relate this requirement to the total consumption of dietary solids and also to report observations of pyridoxine deficiency symptoms that appear in pigs which are inadequately supplied with this vitamin.

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EXPERIMENTAL

Eighty-nine baby pigs were used in 4 trials. Data from only three of these are presented because satisfactory growth rate was not obtained in the positive control group in one of the trials. In each experiment, pigs were taken from the sow at three to 5 days of age and were individually fed a synthetic-milk diet used by Miller et al. ('54) except that pyridoxine was absent from the diet and riboflavin replaced it in an equal amount. The environmental conditions and the length of the depletion-adjustment period were similar to those reported by Miller et al. ('54, '55) and by Stothers et al. ('55).

On the basis of the work of Wintrobe et al. ('43) and of Moustgaard ('53), the minimum pyridoxine requirement was judged not to exceed 2 mg of vitamin B_6 per kilogram of dietary solids intake with the probability that it did not exceed one-half this amount. Consequently, concentrations of 0, 0.5, 0.75, 1.0 and 2.0 mg of pyridoxine per kilogram of solids were used in the diets of 5 lots of pigs which had been assigned at the end of the depletion-adjustment period on the bases of sex, size and litter.

Feedings were made regularly 5 times a day early in the experiment and later on 4 times daily. The amount of feed was generally limited only during the first week of the experiment. Pigs were weighed every 4th day.

Blood samples were taken weekly from an ear vein for red cell counts, white cell counts, white cell differentiation and hemoglobin determinations. Blood samples were taken from the anterior vena cava at the end of the experimental period for serum protein electrophoresis study. Serum samples were obtained after formation of the clot by centrifugal separation. These samples were frozen and stored and, subsequently, separation of the serum proteins was made using the Durrum ('50) type, Spinco, Model R paper electrophoresis apparatus. Total serum protein was determined by the biuret method of Weichselbaum ('46).

Urine collections were made weekly to determine xanthurenic acid excretion values. Collections were made over an 8- to 12-hour period. Before being placed into metabolism cages for this collection, pigs received an oral dose of DL-tryptophan amounting to 100 mg per kilogram of body weight. Urine xanthurenic acid concentration determinations were made using the method of Wachstein and Gudaitis ('52).

As in previously cited work by the present authors, gross and microscopic post mortem examinations were made on all pigs that died, those which were killed in extremis and those positive control pigs which were sacrificed to be used as comparative standards. Various organ weights were also taken and recorded. All data obtained on individual pigs throughout the experiment were grouped and statistically analyzed using the methods of Snedecor ('46) for analyzing single and multiple classification variance.

RESULTS AND DISCUSSION

The combined results of the three feeding trials pertaining to pig growth and feed consumption are presented in table 1. Pigs from all lots receiving pyridoxine gained significantly more rapidly and efficiently than pigs from the lot receiving no dietary pyridoxine. The daily rate of gain of pigs receiving 0.75 mg or more of vitamin B_6 per kilogram of dietary solids was 11 to 14% greater and their daily solids intake significantly greater than for those pigs which received 0.5 mg of vitamin B_6 per kilogram of solids.

The pigs which received no pyridoxine ate and gained normally for the first two weeks of experimental feeding. Then there was a gradual loss of appetite and the rate of gain dropped. Most of these pigs lost weight during the final week of the trial. A typical example of a pyridoxine-deficient pig is shown in figure 1. Vomiting with expulsion of copious amounts of a thick yellowish-green fluid occurred occasionally during the third and 4th weeks of experimental feeding. Epileptiform seizures were observed frequently in

the deficient pigs from the third week until the end of the trial. On every observed occasion in these trials the seizures occurred immediately prior to, during, or just after feeding. Although the seizure appeared spontaneously, its onset seemed to be stimulated by the excitement or the anxiety of the feeding experience. Neurochemical studies by Tower ('56) on samples excised from epileptogenic foci in human cerebral cortex have

TABLE 1

Response of baby pigs to synthetic milk diets containing different levels of pyridoxine [1]

	LEVEL OF PYRIDOXINE IN DIET, IN MG/KG SOLIDS				
	0	0.5	0.75	1.0	2.0
Lot number	1	2	3	4	5
Number of pigs	12	11	11	12	12
Days on test	32	32	32	32	32
Av. initial wt., lbs.	4.10±0.13 [2]	4.19±0.16	4.34±0.15	4.10±0.11	3.94±0.15
Av. final wt., lbs.	8.16±0.32	15.64±0.68	17.45±0.63	16.94±0.38	16.74±0.37
Av. daily gain, lbs.[3]	0.13±0.01	0.36±0.02	0.41±0.02	0.40±0.01	0.40±0.01
Av. daily solids consumed, lbs.[4]	0.21±0.01	0.36±0.01	0.40±0.01	0.39±0.01	0.39±0.01
Solids per lb. gain, lbs.[5]	1.66±0.09	1.00±0.03	0.98±0.03	0.98±0.02	0.99±0.02

[1] Pigs were taken from the sow when 3 to 5 days old and placed on a depletion diet containing no pyridoxine for 4 days. Pigs were then assigned to lots and started on the feeding trial.

[2] Standard error of mean.

[3] Pigs from all lots receiving pyridoxine gained significantly faster ($P = 0.01$) than lot 1 pigs.

[4] Pigs from all lots receiving pyridoxine consumed significantly more solids ($P = 0.01$) than lot 1 pigs.

Pigs from lots 3, 4, and 5 consumed significantly more solids ($P = 0.05$) than lot 2 pigs.

[5] Pigs from all lots receiving pyridoxine were significantly more efficient ($P = 0.01$) in food utilization than pigs from lot 1.

suggested that focal cortical seizures may be due to a biochemical lesion, particularly in the metabolism of glutamic acid. Epileptiform seizures and vomiting were observed in a few of the pigs of lot 2, but none was observed in any of the pigs receiving 0.75 mg or more of vitamin B_6 per kilogram of solids.

A summary of the weekly blood hemoglobin determinations is made in table 2. An increasing degree of hypochromia

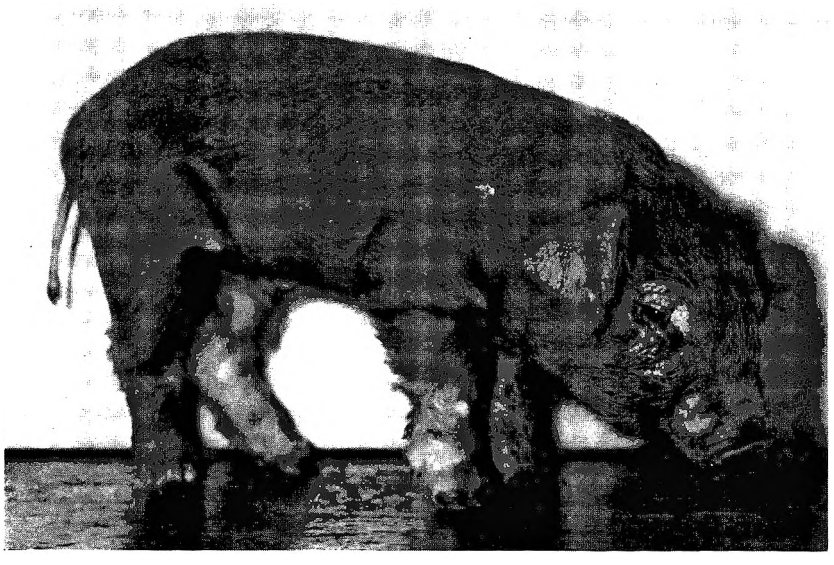

Fig. 1 A pyridoxine-deficient pig 6 weeks of age and weighing only 8 pounds.

developed as the degree of pyridoxine deficiency advanced. This was evident in all pigs receiving 0.5 mg or less of pyridoxine per kilogram of dietary solids. Cartwright and Wintrobe ('48) have shown that the fundamental disturbance causing the hypochromia in pyridoxine-deficient pigs is an inability to synthesize protophorphyrin.

Blood cell studies revealed the presence of pale microcytic erythrocytes from the third week until the end of the trial in pigs receiving less than 0.75 mg of vitamin B_6 per kilogram of solids. Anisocytosis was commonly observed in blood from the deficient pigs also. Data on erythrocyte count presented in table 3 suggest that oligocythemia may be a sig-

TABLE 2

Blood hemoglobin levels[1] *of baby pigs receiving different dietary levels of pyridoxine*

NUMBER OF WEEKS ON EXPERIMENT	LEVEL OF PYRIDOXINE IN DIET, IN MG/KG SOLIDS				
	0	0.5	0.75	1.0	2.0
1	11.5 ± 0.2	11.1 ± 0.3	11.3 ± 0.2	11.4 ± 0.3	11.6 ± 0.3
2	9.9 ± 0.3	11.0 ± 0.4	11.4 ± 0.3	11.0 ± 0.3	11.3 ± 0.3
3 [2]	9.1 ± 0.5	10.9 ± 0.5	12.1 ± 0.3	11.5 ± 0.3	11.1 ± 0.3
4 [2,3]	7.2 ± 0.4	10.2 ± 0.6	12.3 ± 0.4	11.9 ± 0.3	12.3 ± 0.4
5 [2,3]	6.7 ± 0.5	9.3 ± 0.5	11.0 ± 0.5	11.7 ± 0.5	11.7 ± 0.4

[1] Blood hemoglobin level expressed in grams per 100 ml of blood.

[2] Blood from pigs in all lots receiving vitamin B_6 was significantly higher in hemoglobin level ($P = 0.01$) during the third, 4th and 5th weeks than blood from the pigs receiving no B_6.

[3] Pigs from lots receiving 0.75 mg of vitamin B_6 or more per kilogram of solids were significantly higher in blood hemoglobin level ($P = 0.01$) during the 4th and 5th weeks than pigs from the lot receiving 0.5 mg B_6 per kilogram of solids in the milk.

TABLE 3

Initial and final red blood cell and lymphocyte counts of baby pigs receiving different dietary levels of pyridoxine

LEVEL OF PYRIDOXINE	RBC [1]		LYMPHOCYTE [2]	
	Initial	Final [3]	Initial	Final [4]
mg/kg solids				
0	6.61 ± 0.23	4.43 ± 0.38	60 ± 2	37 ± 5
0.5	5.91 ± 0.17	6.10 ± 0.18	57 ± 3	51 ± 4
0.75	6.24 ± 0.20	6.37 ± 0.23	54 ± 4	66 ± 4
1.0	5.95 ± 0.20	6.98 ± 0.28	53 ± 3	61 ± 3
2.0	6.26 ± 0.21	7.05 ± 0.26	62 ± 3	61 ± 3

[1] Red blood cell count expressed in millions per cubic millimeter.

[2] Lymphocyte count expressed as a per cent of the total leukocyte count.

[3] Pigs from all lots receiving pyridoxine had a significantly higher ($P = 0.01$) RBC count than pigs from the lots receiving no pyridoxine. Pigs receiving 1.0 mg of vitamin B_6 or more per kilogram solids in their diet had a significantly higher ($P = 0.05$) RBC count than pigs receiving 0.5 mg B_6 per kilogram solids.

[4] Pigs receiving 0.75 mg of vitamin B_6 or more per kilogram solids had a significantly higher ($P = 0.01$) percentage of lymphocytes than pigs from the lots receiving no B_6. Pigs from the lot receiving 0.5 mg B_6 per kilogram solids had a significantly higher ($P = 0.05$) percentage of lymphocytes than pigs from the lot receiving no B_6, but a significantly lower ($P = 0.05$) percentage of lymphocytes than pigs from all other lots.

nificant factor in the promotion of pyridoxine-deficiency anemia. Dinning and Day ('56) have recently reported a significant increase in erythrocyte count in the pyridoxine-deficient rat.

Total leukocyte counts in pyridoxine-deficient pigs did not differ significantly from those of control pigs at any time during the trial. However, data in table 3 show a significant lymphocytopenia in the vitamin B_6-deficient pigs. There was a corresponding increase in the percentage of neutrophils in the peripheral blood of these animals. Stoerk ('46), Agnew and Cook ('49) and Dinning and Day ('56) have found greatly reduced lymphocyte production in vitamin B_6-deficient rats. Dougherty et al. ('44) have demonstrated the presence of antibodies in lymphocytes in mice and Stoerck and Eisen ('46), Agnew and Cook ('49), Axelrod et al. ('47) and Ludovici et al. ('51) have all observed reduced antibody response in vitamin B_6-deficient rats.

Moustgaard ('53) has reported urine xanthurenic acid excretion to be a very sensitive measure of pyridoxine deficiency in the weanling pig. Data presented in table 4 shows this to be a sensitive test for dietary pyridoxine adequacy in the baby pig also. Inspection of the data indicates that the level of dietary pyridoxine adequacy lies between 0.75 and 1.0 mg of vitamin B_6 per kilogram of solids.

In studying the physiological effects of a nutrient deficiency, the research worker often wonders if the effects are a specific result of the nutrient deficiency or a result of the coincidentally reduced appetite. To test this in the present work, 4 pigs receiving the positive control diet were pair fed with 4 pigs receiving no pyridoxine. The results of this trial are presented in table 5 and demonstrate that the hypochromia, oligocythemia, lymphocytopenia and the improper metabolism of tryptophan observed in the deficient pigs were specific effects of pyridoxine deficiency and did not result from the ensuing anorexia. No evidence of microcytosis or anisocytosis was observed in blood from the pair-fed positive

control pigs nor were epileptiform seizures ever observed in these pigs.

Moustgaard ('53) has observed an impairment of the pyridoxine-deficient pig's ability to produce gamma globulin. This fraction constituted 5% of the total serum protein in the

TABLE 4

Concentration of xanthurenic acid[1] *in the urine from baby pigs receiving different dietary levels of pyridoxine*

	LEVEL OF PYRIDOXINE IN DIET, IN MG/KG SOLIDS				
	0	0.5	0.75	1.0	2.0
Number of pigs tested	8	11	11	8	8
Number of weeks on experimental diet					
1	14 ± 2	12 ± 1	16 ± 2	10 ± 0	9 ± 3
2[2]	29 ± 2	19 ± 3	21 ± 3	12 ± 3	9 ± 3
3[3]	35 ± 4	17 ± 2	15 ± 2	13 ± 1	9 ± 3
4[3]	62 ± 19	26 ± 4	14 ± 2	8 ± 3	5 ± 5
5[3]	127 ± 29	35 ± 3	32 ± 4	6 ± 2	3 ± 2

[1] Concentration of XA expressed in micrograms per milliliter of urine.

[2] Pigs receiving 1.0 mg and 2.0 mg of vitamin B_6 per kilogram of solids in their diet excreted urine containing a significantly lower ($P = 0.01$) concentration of XA than pigs receiving no pyridoxine during the second week of the experiment.

[3] Pigs from the lot receiving no pyridoxine excreted urine containing a significantly higher ($P = 0.01$) concentration of XA than pigs from all other lots during the third, 4th and 5th weeks of the experiment.

TABLE 5

Comparison of performance and certain blood and urine components of positive control pair-fed pigs with negative controls

	LEVEL OF PYRIDOXINE IN DIET, IN MG/KG SOLIDS	
	0	2.0
Number of pigs	4	4
Days on test	32	32
Av. daily gain, lb.	0.11 ± 0.01	0.19 ± 0.01**
Av. daily solids consumed, lb.	0.21 ± 0.01	0.21 ± 0.01
Solids per lb. gain, lb.	1.91 ± 0.20	1.09 ± 0.05**
Blood hemoglobin level, gm/100 ml	7.4 ± 0.6	13.2 ± 0.7 **
Red blood cell count, millions/mm^3	5.41 ± 0.37	7.99 ± 0.67*
Lymphocyte count, % of WBC	38 ± 7	59 ± 6*
Urine XA concentration, μg/ml	72 ± 29	5 ± 5*

* Significantly different from negative controls ($P = 0.05$); ** ($P = 0.01$).

vitamin B_6-deficient pigs in his work. In the present study the gamma globulin fraction was found to be increased. The alpha globulin fraction was also increased and the albumin fraction was decreased in the serum of pigs receiving no pyridoxine in the diet. A summary of these data is presented in table 6. The amount of total serum protein was not significantly altered by the pyridoxine deficiency in this study.

TABLE 6

Total serum protein concentration and electrophoretic distribution of serum proteins in baby pigs receiving different dietary levels of pyridoxine

	LEVEL OF PYRIDOXINE IN DIET, IN MG/KG SOLIDS				
	0	0.5	0.75	1.0	2.0
Number of pigs	5	11	11	8	5
Total serum protein, gm/100 ml	5.75 ± 0.26	5.80 ± 0.18	5.60 ± 0.30	5.55 ± 0.22	5.64 ± 0.45
Serum protein distribution, % of total					
Albumin	52.9 ± 2.3	44.7 ± 0.9**	44.6 ± 1.0**	44.4 ± 2.1**	42.0 ± 1.7**
α Globulin	24.7 ± 1.7	27.8 ± 1.1**	25.4 ± 0.9**	27.2 ± 1.5**	27.9 ± 0.6**
β Globulin	16.8 ± 0.3	18.9 ± 0.8	20.9 ± 0.8*	18.8 ± 0.7	20.3 ± 1.5*
γ Globulin	15.3 ± 1.1	8.6 ± 0.8**	9.1 ± 0.4**	9.6 ± 1.2**	9.7 ± 0.8**

* Significantly different from sera of pigs receiving no pyridoxine ($P = 0.05$); ** ($P = 0.01$).

The presence of a generalized subcutaneous edema in all deficient pigs on which post mortem studies were made was probably a manifestation of the low serum albumin values present in these animals. Relative values of the serum protein fractions and the values for serum total protein obtained in this study in pigs receiving pyridoxine were quite similar to those obtained by Foster et al. ('51) for fibrinogen-free plasma from pigs at weaning age.

The only consistent gross finding in post mortem studies of the deficient pig was the previously mentioned anasarca.

Occasionally there was an excessive amount of fluid in the pericardial sac and both the liver and heart appeared somewhat fatty. Microscopic examination revealed fatty degeneration in the liver of some of the pigs which received no pyridoxine. All pigs receiving no pyridoxine and some receiving 0.5 mg of vitamin B_6 per kilogram of solids showed a brownish pigment in the splenic pulp. This pigment, probably hemosiderin, was also present in the Kupffer cells of the liver of some of these pigs.

The presence of lesions in the nervous system was questionable. No changes were noted in the brain or spinal cord. Myelin degeneration in the sciatic nerve was not observed from Weil's ('45) stain. An occasional cell in the dorsal root ganglion of deficient pigs was somewhat atrophied and occasionally showed slight chromatolysis. Other investigators, Follis and Wintrobe ('45), Wintrobe et al. ('42, '43) and Swank and Adams ('48) have reported lesions in the spinal cord and in the peripheral nerves. The reason that these lesions were not demonstrated in the present investigation lies, perhaps, in the fact that the pigs were not on the deficient diets for as long a period of time as in the previously reported work on older pigs.

A summary of the data on the weights of heart, liver, kidneys, thyroid and adrenals, obtained from the post-mortem studies, is given in table 7. In the case of the pyridoxine-deficient pigs, each of these critical organs comprised a greater percentage of the body weight than was true of either the inanition- or full-control animals. These differences were statistically significant for the kidneys and the adrenals. Agnew ('55) has reported cardiac, renal and hepatic hypertrophy in pyridoxine-deficient rats.

Two of the pigs which were receiving no pyridoxine during the 32-day experimental period received pyridoxine therapy and recovered. Both of these pigs were manifesting severe pyridoxine deficiency at the end of the feeding period and were placed on the positive control level of dietary pyri-

doxine intake. In addition, one of these pigs received a single 50 mg intraperitoneal injection of pyridoxine hydrochloride. Within a few days the anorexia subsided and normal gains followed in both pigs. By the end of the 24-day recovery period both pigs were making excellent gains. Neither of the pigs had a recurrence of the epileptiform seizures after treatment commenced. Blood components quickly returned to normal and urine xanthurenic acid excretion dropped sharply. One of the pigs was sacrificed at the end of the recovery period and post mortem examination revealed no abnormalities.

TABLE 7

Relation of certain critical organ weights to total body weight in normal, pyridoxine-deficient and pair-fed control baby pigs

	CONTROLS	PYRIDOXINE-DEFICIENT	PAIR-FED CONTROLS
Number of pigs sacrificed	4	9	3
Relative organ weights [1]			
Heart	0.52 ± 0.01	0.63 ± 0.07	0.59 ± 0.04
Liver	2.90 ± 0.10	3.95 ± 0.50	2.72 ± 0.23
Kidneys	0.62 ± 0.02	1.07 ± 0.12**	0.56 ± 0.02
Thyroid	0.009 ± 0.001	0.016 ± 0.002	0.009 ± 0.002
Adrenals	0.016 ± 0.001	0.035 ± 0.005*	0.015 ± 0.002

[1] Per cent of total body weight.

* Significantly greater than corresponding positive control relative organ weight ($P = 0.05$); ** ($P = 0.01$).

SUMMARY

Levels of pyridoxine supplementation constituting 0, 0.5, 0.75, 1.0, and 2.0 mg per kilogram of solids in a synthetic milk diet were used to determine the requirement of the pig for this vitamin. An analysis of the growth and feed consumption data indicates that the 0.5 mg level is nearly adequate. However, data obtained on blood hemoglobin, red blood cell and lymphocyte counts indicate that the minimum requirement is not less than 0.75 mg of pyridoxine per kilogram of solids. With respect to urinary xanthurenic acid, however, the level of 0.75 mg of pyridoxine is inadequate. It

would thus appear that when a more nearly total consideration of the pig's well-being is given, the requirement is greater than 0.75 mg but probably less than 1.0 mg of pyridoxine per kilogram of solids. Classical deficiency symptoms were observed in those pigs receiving no pyridoxine. The performance of pair-fed controls indicated that the effects on the blood and urine components found in deficient pigs were specific effects of pyridoxine deficiency and not effects of the resulting inanition.

Serum total protein values were not affected by pyridoxine deficiency but the relative values of certain serum protein fractions were altered. Evidence of renal and adrenal hypertrophy are presented. Therapeutic treatment of pyridoxine-deficient pigs brought rapid recovery.

LITERATURE CITED

AGNEW, L. R. C. 1955 Cardiac, renal and hepatic hypertrophy in pyridoxine-deficient rats. Proc. Soc. Exp. Biol. Med., *90:* 452.

AGNEW, L. R. C., AND R. COOK 1949 Antibody production in pyridoxine-deficient rats. Brit. J. Nutrition, *2:* 321.

AXELROD, A. E., B. B. CARTER, R. H. MCCOY AND R. GEISINGER 1947 Circulating antibodies in vitamin-deficiency states. I. Pyridoxine, riboflavin and pantothenic acid deficiencies. Ibid., *66:* 137.

CARTWRIGHT, G. E., AND M. M. WINTROBE 1948 Studies on free erythrocyte protoporphyrin, plasma copper and plasma iron in normal and in pyridoxine-deficient swine. J. Biol. Chem., *172:* 557.

CHICK, H., T. F. MACRAE, A. J. P. MARTIN AND C. J. MARTIN 1938 The water-soluble B-vitamins other than aneurin (vitamin B_1), riboflavin and nicotinic acid required by the pig. Biochem. J., *32:* 2207.

DINNING, J. S., AND P. L. DAY 1956 Vitamin B_6 and erythropoiesis in the rat. Proc. Soc. Exp. Biol. Med., *92:* 115.

DOUGHERTY, T. F., J. H. CHASE AND A. WHITE 1944 The demonstration of antibodies in lymphocytes. Ibid., *57:* 295.

DURRUM, E. L. 1950 A microelectrophoretic and microionophoretic technique. J. Am. Chem. Soc., *72:* 2943.

FOLLIS, R. H., JR., AND M. M. WINTROBE 1945 A comparison of the effects of pyridoxine and pantothenic acid deficiencies on the nervous tissues of swine. J. Exp. Med., *81:* 539.

FOSTER, J. F., R. W. FRIEDELL, D. CATRON AND M. W. DIECKMAN 1951 Electrophoretic studies on swine. III. Composition of baby pig plasma and sow's whey during lactation. Arch. Biochem. and Biophys., *31:* 104.

HUGHES, E. H., AND R. L. SQUIBB 1942 Vitamin B_6 (pyridoxine) in the nutrition of the pig. J. An. Sci., *1:* 320.

LEHRER, W. P., JR., A. C. WIESE, P. R. MOORE AND M. E. ENSMINGER 1951 Pyridoxine deficiency in baby pigs. Ibid., *10:* 65.

LUDOVICI, P. P., A. E. AXELROD AND B. B. CARTER 1951 Circulating antibodies in vitamin-deficiency states. Pantothenic acid and pyridoxine deficiencies. Proc. Soc. Exp. Biol. Med., *76:* 665.

MILLER, E. R., R. L. JOHNSTON, J. A. HOEFER AND R. W. LUECKE 1954 The riboflavin requirement of the baby pig. J. Nutrition, *52:* 405.

MILLER, E. R., D. A. SCHMIDT, J. A. HOEFER AND R. W. LUECKE 1955 The thiamine requirement of the baby pig. Ibid., *56:* 423.

MOUSTGAARD, J. 1953 The vitamin B requirement of young pigs to ensure the best possible gain and food utilization. Reprint from the congress report of the VIth International Congress of Animal Husbandry.

MOUSTGAARD, J., P. MØLLER AND G. THORBEK 1952 Undersøgelser over B-Vitaminernes Betydning for Svin. V. Svinenes Behor at Pyridoxin (B_6) for Maximal Vaekst og Foderudnyttelse. *258. Ber. fra Forsøgslaboratoriet,* København.

SNEDECOR, G. W. 1946 Statistical Methods. Fourth Edition. Iowa State College Press, Ames.

STOERK, H. C. 1946 Effects of calcium deficiency and pyridoxine deficiency on thymic atrophy. Proc. Soc. Exp. Biol. Med., *62:* 90.

STOERK, H. C., AND H. N. EISEN 1946 Suppression of circulating antibodies in pyridoxine deficiency. Ibid., *62:* 88.

STOTHERS, S. C., D. A. SCHMIDT, R. L. JOHNSTON, J. A. HOEFER AND R. W. LUECKE 1955 The pantothenic acid requirement of the baby pig. J. Nutrition, *57:* 47.

SWANK, R. L., AND R. D. ADAMS 1948 Pyridoxine and pantothenic acid deficiency in swine. J. Neuropath. and Exp. Neurology, *7:* 274.

TOWER, D. B. 1956 Neurochemical aspects of pyridoxine metabolism and function. J. Clin. Nutrition, *4:* 329.

WACHSTEIN, M., AND A. GUDAITIS 1952 Detection of vitamin B_6 deficiency. Utilization of an improved method for rapid determination of xanthurenic acid in urine. J. Lab. Clin. Med., *40:* 550.

WEICHSELBAUM, T. E. 1946 An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Am. J. Clin. Path., *16 (techn. sec.):* 40.

WEIL, A. 1945 Textbook of Neuropathology. Second Edition. Grune and Stratton, New York.

WINTROBE, M. M., M. H. MILLER, R. H. FOLLIS, JR., H. J. STEIN, C. MUSHATT AND S. HUMPHREYS 1942 Sensory neuron degeneration in pigs. IV. Protection afforded by calcium pantothenate and pyridoxine. J. Nutrition, *24:* 345.

WINTROBE, M. M., R. H. FOLLIS, JR., M. H. MILLER, H. J. STEIN, R. ALCAYAGA, S. HUMPHREYS, A. SUKSTA AND G. C. CARTWRIGHT 1943 Pyridoxine deficiency in swine, with particular reference to anemia, epileptiform convulsions and fatty liver. Bull. Johns Hopkins Hosp., *72:* 1.

THE EFFECTS OF DIFFERENT FOOD FATS ON SERUM CHOLESTEROL CONCENTRATION IN MAN [1]

JOSEPH T. ANDERSON, ANCEL KEYS AND FRANCISCO GRANDE
Laboratory of Physiological Hygiene, University of Minnesota, Minneapolis, and The Hastings State Hospital, Hastings, Minnesota

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Though a simple reduction in the fat content of the average American diet usually produces a prompt fall in the serum cholesterol concentration (Keys et al., '50; Mellinkoff et al., '50; Stark, '50; Groen et al., '52; Mayer et al., '54; Keys et al., '55), synthetic or artificial diets containing large amounts of some vegetable oils may also depress the serum cholesterol, at least in short-time experiments (Kinsell et al., '52; Ahrens et al., '54; Bronte-Stewart et al., '56). Controlled comparisons of different food fats in normal human diets of ordinary foodstuffs have been notably lacking and the present paper reports such comparisons involving butterfat, corn (maize) oil, cottonseed oil, coconut oil, olive oil, sunflower seed oil and sardine oil fed at levels representing 30 to 35% of the total calories. In addition, the effects of these fats are compared with those of ordinary American and with low-fat diets in which fats contribute, respectively, around 40 and 9 to 16% of total calories.

[1] The studies reported here were aided by research grants from the National Dairy Council, Chicago, the Minnesota Heart Association, the Mental Health Research Fund of the Minnesota State Department of Public Welfare and the Schweppe Foundation, Chicago.

PROCEDURE

Subjects and their regimen. The subjects were schizophrenic men, chiefly of the hebephrenic and paranoid types, long resident at the Hastings State Hospital. They were judged to be physically and metabolically "normal" on the basis of detailed physical and laboratory examinations and were selected as being cooperative and relatively stable in their emotional condition. Their ages ranged from 32 to 62 years, the averages being 39.0, 44.7, 46.2 and 47.6 years in experiments C, F, H, and J, respectively. Within each experiment the men were assigned to sub-groups whose regimens differed only in regard to the diet, and these sub-groups were matched in regard to age, relative obesity and habitual degree of activity.

The ancestry of these men was almost exclusively from the British Isles and Northern Europe. Their heights ranged from 5 feet 3 inches (160 cm) to 6 feet 2 inches (188 cm), the average being 5 feet 8.3 inches (173.5 cm). None of the men was grossly obese or emaciated but they tended to be somewhat thinner than men in the general population of Minnesota, their average being about 9 pounds (4 kg) less than the average for business and professional men of the same age and height in Minneapolis.

During the entire period of each experiment the men were housed and fed in the Metabolic Research Unit of the Hastings State Hospital where they were under 24-hour a day surveillance, during their standardized outdoor recreation as well as within the locked building, by special resident aides in addition to the regular hospital staff. Meals were prepared and served in the diet kitchen of the Metabolic Unit under the supervision of a special dietitian whose responsibilities were limited to these subjects. No other foods were permitted.

The regimen was standardized to maintain physical activity and metabolic balance as constant as possible throughout the period of each experiment. The caloric need for balance for each man was estimated during a month of preliminary testing

before the start of each experiment proper and the size of the food servings for each man was adjusted accordingly. Nude body weights were recorded at least every week and these measurements were the basis for further adjustments in the calorie values of the diets.

In the few instances when men became uncooperative or ill they were dropped from the test group and all data on these individuals were excluded from subsequent analysis. Weight change of as much as 2 kg in a month or 3 kg over an entire experiment was also ground for excluding men, and their data, from further consideration. For these several reasons we excluded a total of 27 out of 159 men in the entire series of experiments, 10 of the exclusions occurring in the first experiment (C) of the series. Improved selection and management of the men resulted in losing only two out of 26 men in both experiments H and J; this probably is close to an irreducible minimum for experiments averaging 5 to 6 months each in duration, including before and after controls.

The diets. The standard Hastings State Hospital diet, planned and prepared under the supervision of competent dietitians, conforms to the ordinary American pattern and provides an abundance of meat, milk, butter, vegetables and fruits. As served in measured portions in the Metabolic Unit, this is termed the "House Diet" and provides about 90 gm of proteins and 140 gm of fats (one-third being butterfat) per 3200 Cal. The cholesterol content of this standard "House Diet" varies from 600 to 800 mg daily, the general average of other nutrients being approximately: vitamin A, 6,000 to 10,000 I.U.; thiamine, 2.2 mg; riboflavin, 2.3 mg; niacin, 22 mg; pyridoxine, 2.2 mg; inositol, 900 mg; choline, 2500 mg; ascorbic acid, 100 to 170 mg; tocopherols, 18 mg; phytosterols, 90 mg; iron, 12 mg; calcium, 1200 mg; poly-unsaturated fatty acid glycerides, 10 gm, of which about 8 gm represents linoleic acid glycerides.

The men were maintained on this "House Diet" for at least 4 weeks before the start of each experiment with the various experimental diets. The basis of all of the experi-

mental diets was a low-fat diet in which one or the other fat could be isocalorically substituted for carbohydrate, replacing equivalent calories in sugar, jelly, bread, rice and potatoes. The low-fat diet provided from 9 to 12% of calories from total fats and 10 to 12% of calories from proteins in the several experiments. Other characteristics of a typical low-fat diet, and this diet with test fats, are indicated in table 1. The test

TABLE 1

Nutrients in the experimental diets

(Calories, proteins and fats are the averages of the amounts eaten on 4 representative days in each period. The other items represent amounts served on a typical day in each period.)

CONSTITUENT	DIETS	
	Low fat	Low fat + experimental fat
Calories	3100	3100
Protein, gm	94	94
Total fats, gm	38	131
Saturated fats, gm	17	40 [1]
Linoleic acid (as glycerides) gm	4	51 [1]
Cholesterol, mg	660	660
Phytosterols, mg	67	366 [1]
Thiamine, mg	2.4	2.4
Riboflavin, mg	3.1	3.1
Niacin, mg	18	18
Pyridoxin, mg	1.7	1.7
Ascorbic acid, mg	160	160
Vitamin A, I.U.	18000	18000
Tocopherols, mg	11	120 [1]

[1] Experimental fat = cottonseed oil.

diets were planned to provide a level of 50 gm of test fat per day in experiment C, and 100 gm per day in experiments F, H and J, so that total fats would account for about 25% of total calories in experiment C and close to 40% in the other experiments.

In each experiment the period on each diet was 4 weeks, except in experiment J where the dietary periods were two weeks each. During these dietary periods all foods were measured out for each man and record was kept of all extra

portions served to satisfy the wants of the men and to maintain weight equilibrium. Plate waste was recorded on 4 representative days of each dietary period (three days in experiment J) and the average food consumption computed for these days.

Fats in the diets. Characteristics of the fats in the diets are summarized in table 2. These fats and oils were purchased in large lots, to ensure uniformity throughout each experiment, and were considered to be typical of good commercial

TABLE 2

Characteristics of the fats and oils used in the present experiments

(The iodine values are those found in the fats as used. The fatty acid values are computed from the data given [1] for samples of those fats having comparable iodine values. For the "House Diet" fat, fatty acid composition was computed from values for fats making up about 95% of the total fat.)

FAT	IODINE VALUE	% OF FATTY ACIDS		
		Saturated	Mono-ethenoid	Poly-ethenoid
Butterfat	32	57	39	4
Olive oil	85	12	80	8
Cottonseed oil [2]	108	25	25	50
Corn oil [3]	120	12	37	51
Sunflower seed oil [4]	131	10	28	62
Coconut oil [5]	3	97	3	0
Sardine oil [6]	188	23	23	54
"House Diet" fat [7]	50	48	45	7

[1] Fatty acid analyses were computed from data given by A. E. Bailey, Industrial Oil and Fat Products, Interscience, New York, 1951; E. W. Eckey, Industrial Fats and Oils, Reinhold, New York, 1954; and T. P. Hilditch, The Chemical Constitution of Natural Fats, 3rd Ed. Wiley, New York, 1956.

[2] Wesson brand.

[3] Mazola brand.

[4] Saflo brand, Co-op Vegetable Oil, Altona, Manitoba, Canada.

[5] The coconut oil used here was "Hydrol," a hydrogenated product of Durkee's Famous Foods, Inc.

[6] From Van Camp Sea Food Co., Terminal Island, California. Composition estimated from data on the degree of unsaturation of the various fractions (see Hilditch, '56).

[7] The iodine value computed from this fatty acid composition agrees with the tabulated iodine value which was measured directly on the mixed fat extracted from the entire diet.

grades in the American market.[2] The "House Diet" fat was obtained by extraction with ethyl ether, after drying of the combined servings from several typical meals.

The iodine values in table 2 were measured in this laboratory. The fatty acid composition values given in table 2 are primarily those in the literature for commercial samples of these oils that exhibit similar iodine values.

Analytical methods. Blood samples were drawn in the morning, with the men at rest before eating their first meal of the day, on two occasions near the end of each dietary period. The serum was analyzed, in duplicate, for total cholesterol, by our version (Anderson and Keys, '56) of the method of Abell et al. ('52). In addition, the cholesterol distribution between alpha and beta lipoproteins was separately measured (Anderson and Keys, '56). In experiment F, about two-thirds of the samples were subjected to fractionation of the lipoproteins, both by cold ethanol and by paper electrophoresis; in experiments J and H all samples were fractionated by paper electrophoresis.

Experiment C: low-fat, olive oil and cottonseed oil. Experiment C, the first experiment in this series, was designed to determine the effects on the serum cholesterol concentration, in periods of 4 weeks on each diet, of the isocaloric substitution of a moderate amount (50 gm daily) of olive oil and of cottonseed oil for carbohydrate in a low-fat diet.

The main data of experiment C are summarized in table 3. Total cholesterol concentrations were significantly lower on the three experimental diets than on the "House Diet." The mean differences for total cholesterol, in milligrams per 100 ml of serum, with the standard errors computed for the changes of the individual men, were: House-low-fat, 31.2 ± 5.9; House-olive oil, 15.8 ± 4.5; House-cottonseed oil, 20.6 ± 5.2. There

[2] The cottonseed oil was "Wesson" brand; the corn oil was Mazola; the sunflower seed oil was the standard product of Co-op Vegetable Oils, Altona, Manitoba, Canada; the sardine oil was a refined product obtained from Van Camp Sea Food Co., Terminal Island, California; the coconut oil was a hydrogenated product sold under the name "Hydrol" by Durkee's Famous Foods.

was no significant difference between the effects of olive oil and those of cottonseed oil but the low-fat diet was more effective than the other experimental diets in lowering the serum cholesterol concentration from the "House Diet" level, particularly in the second low-fat diet period. Unfortunately, there was poor agreement between the two low-fat periods and the variability of the individuals was great. Nevertheless, by using all the observed cholesterol values on olive oil and cottonseed oil and comparing them with the values on the

TABLE 3

Experiment C.[1] *Mean serum total cholesterol concentrations at the ends of the experimental diet periods, together with the average fat intakes*

DIET	FAT INTAKE		CHOLESTEROL
	gm/day	*% Cal.*	*mg %*
"House," mean	144.6	40.9	229.4 ± 9.8[2]
Low-fat, mean	28.4	9.5	198.2 ± 7.0
Olive oil	80.7	25.2	213.6 ± 9.1
Cottonseed oil	79.6	25.1	208.8 ± 10.2

[1] The 16 men were divided into two groups (A and B) of 8 each. Dietary periods were 4 weeks and the sequence was for group A: "House Diet," low-fat, olive oil, cottonseed oil, low-fat and "House Diet," and for group B: "House Diet," low-fat, cottonseed, olive, low-fat and "House Diet."

[2] Mean and standard error of the mean.

two low-fat diets statistical analysis of the individual differences indicated that the cholesterol concentration on the oil diets was significantly higher than that in the low-fat periods at the 0.05 probability level. The experience of experiment C enabled us to achieve much better control and standardization of the men and their diets in the subsequent experiments (F, H and J).

Final computation of the calorie values of the foods actually eaten on the several diets showed average values of 2883 and 2862 Cal. on the olive and cottonseed oil diets and a lower average of 2661 Cal. on the low-fat diet. The average body weight change during 4 weeks on the olive oil diet was + 0.4 kg and on the cottonseed oil diet it was — 0.2 kg; during the

low-fat diet periods there was an average loss of 0.9 kg. Accordingly, it is possible that some part of the cholesterol reduction on the low-fat diet may have been associated with a small negative calorie balance.

In experiment C, measurements of the S_f 12–20 lipoproteins were also made on the serum, using the method of Gofman et al. ('50) as described by Anderson et al. ('57). While the average values for S_f 12–20 fell from the house diet average on all three experimental diets, the differences were not statistically significant: low-fat, -3.6 ± 2.7; olive oil, -1.4 ± 2.3; cottonseed oil, -5.4 ± 3.1 mg per 100 ml.

TABLE 4

Experiment F design, 9 men in each group

(Total fat and fat of vegetable origin, in grams per day, are given within parentheses.)

GROUP	WEEKS 0–4	WEEKS 5–8	WEEKS 9–12	WEEKS 13–16
A	"House" (135, 14)	Low fat (39, 5)	Cottonseed (135, 100)	Butter (133, 5)
B	"House" (134, 14)	Low fat (38, 5)	Butter (126, 5)	Cottonseed (130, 95)

Experiment F: low-fat, cottonseed oil, butterfat. The design of experiment F is shown in table 4 which also gives the average daily intakes of total fat and of fat of vegetable origin for both groups for each of the dietary periods. The quantities of fats interchanged in these diets were about twice those used in experiment C and provided about 28% of the total calories. The serum total cholesterol results are summarized in table 5.

On both the low-fat and the cottonseed oil diets there was a very substantial reduction in the concentration of total cholesterol in the serum. The indication of a slightly greater effect of the low-fat diet does not approach statistical significance. There was no statistically significant difference between the values on the house diet and those on the butterfat diet.

The distribution of serum cholesterol between the alpha and beta lipoprotein fractions in experiment F is discussed, below, together with corresponding data from the other experiments.

Experiment H: low-fat, cottonseed, corn, hydrogenated coconut oils. Table 6 shows the design of experiment H and the average body weights at the end of each dietary period for the 4 groups of men. The average intakes of proteins, fats and cholesterol of groups A and B and of groups C and

TABLE 5

Serum cholesterol differences in the diets of experiment F, 18 men

(The experimental variable was approximately 100 gm of fat. Serum samples were taken at the ends of the 4-week diet periods. Differences in serum cholesterol concentration [mg per 100 ml] between the dietary periods being compared were obtained by subtracting the value on the second diet from that on the first. Mean serum cholesterol on the "House Diet" was 231.8 mg per 100 ml with a standard error of 11.1 mg.)

DIET COMPARISON	MEAN DIFFERENCE	S.E. OF MEAN
Butterfat *minus* "House"	+ 3.0	± 4.7
Butterfat *minus* Low-fat	+ 31.8	± 4.2
Butterfat *minus* Cottonseed oil	+ 31.4	± 4.9
Cottonseed *minus* Low-fat	+ 0.4	± 4.2

TABLE 6

Experiment H design

(Four groups of men, 4 successive periods of 4 weeks each. Mean body weights, in kilograms, for the end of each diet period are given in parentheses.)

PERIOD	GROUP A (6 men)	GROUP B (7 men)	GROUP C (6 men)	GROUP D (6 men)
1	"House" (65.0)	"House" (71.8)	"House" (71.0)	"House" (68.0)
2	Low-fat (64.8)	Low-fat (71.5)	Low-fat (71.2)	Low-fat (68.0)
3	Cottonseed (65.4)	Corn (71.2)	Cottonseed (71.6)	Coconut (67.5)
4	Corn (65.7)	Cottonseed (71.5)	Coconut (71.3)	Cottonseed (68.6)

D on the several diets are given in table 7. The experimental variable was a quantity of oil supplying about 28% of the total calories.

The results for serum total cholesterol are summarized in table 8. As before, both low-fat and cottonseed oil diets produced highly significant decreases in the serum cholesterol as compared with the values on the "House Diet," but the

TABLE 7

Average nutrients eaten on 4 diets in experiment H

GROUPS OF MEN	NUTRIENT	DIETS			
		Low-fat	Cottonseed oil	Corn oil	Coconut oil
A, B	Protein, gm/day	104	99	99	..
	Butterfat, gm/day	11	10	10	..
	Vegetable fat, gm/day	5	98	97	..
	Total fat, gm/day	40	131	130	..
	Cholesterol, mg/day	313	293	295	..
C, D	Proteins, gm/day	104	99	..	98
	Butterfat, gm/day	11	11	..	10
	Vegetable fat, gm/day	6	101	..	99
	Total fat, gm/day	43	136	..	133
	Cholesterol, mg/day	330	308	..	305

TABLE 8

Serum cholesterol differences between the diets of experiment H

(The dietary variable was approximately 100 gm of oil. Differences in total cholesterol concentrations, in milligrams per 100 ml, for the same individuals at the ends of different dietary periods were computed as described in table 5. Mean values of total serum cholesterol on the "House Diet": group A, B = 221.3, group C, D = 213.3 mg per 100 ml.)

DIET COMPARISON		GROUPS	NO. OF MEN	MEAN DIFFERENCE	S.E. OF MEAN
"House"	*Minus* Cottonseed	A, B, C, D	25	+ 38.6	± 5.1
"House"	*Minus* Low-fat	A, B, C, D	25	+ 33.7	± 4.4
Low-fat	*Minus* Cottonseed	A, B, C, D	25	+ 4.9	± 4.0
"House"	*Minus* Corn	A, B	13	+ 61.1	± 5.4
Low-fat	*Minus* Corn	A, B	13	+ 26.1	± 3.9
Cottonseed	*Minus* Corn	A, B	13	+ 19.3	± 4.2
Coconut	*Minus* "House"	C, D	12	+ 11.0	± 2.8
Coconut	*Minus* Cottonseed	C, D	12	+ 46.1	± 6.8

corn oil diet produced an even greater decrease. Further it is apparent that the values on corn oil are significantly lower than those on both the low-fat and the cottonseed oil diets. Finally, the cholesterol values on the hydrogenated coconut oil diet were significantly higher than those on the "House Diet."

Experiment J, including sunflower and sardine oils. The design of experiment J is shown in table 9 together with the

TABLE 9

Experiment J design

(Groups W and X contained 7 men each; groups Y and Z, 6 men each. Each dietary period consisted of two weeks and the parentheses in the table enclose the average values, in grams per day, of total fats, and the part of the fats composed of polyethenoid fatty acids for the several dietary periods. The last line gives the average daily calorie consumption for the entire experimntal period for each of the 4 groups.)

PERIOD	GROUP			
	W	X	Y	Z
1	"House" (156, 11)	"House" (159, 11)	"House" (151, 10)	"House" (153, 10)
2	Low-fat (39, 3)	Low-fat (39, 3)	Butter (130, 6)	Butter (132, 6)
3	Butter (138, 6)	Butter (136, 6)	Butter (122, 6)	Butter (127, 6)
4	Olive (140, 10)	Corn (139, 58)	Olive (132, 10)	Cottonseed (128, 49)
5	Corn (140, 59)	Olive (139, 10)	Cottonseed (128, 50)	Olive (127, 10)
6	Corn (141, 59)	Sunflower (139, 10)	Corn (128, 56)	Sardine (134, 55)
7	Sunflower (141, 64)	Corn (135, 56)	Sardine (126, 52)	Corn (131, 54)
8	Butter (142, 6)	Butter (138, 6)	Butter (130, 5)	Butter (127, 5)
9	"House" (156, 11)	"House" (159, 11)	"House" (151, 10)	"House" (153, 10)
All periods	3310 Cal.	3370 Cal.	2930 Cal.	3050 Cal.

average values for the fats eaten by the 4 groups of men in each of the 9 dietary periods. The effort was made to provide for the comparison of the cholesterol levels on each of 6 diets differing only in the kind of fat which made up approximately 28% of the total calories, as well as on the same basic diet with carbohydrate replacing the test fats (low-fat diet) and on the "House diet." The net result is that unless it is assumed (1) that the groups are ideally matched, (2) that the effects of a preceding diet are completely eliminated in two weeks on another diet, and (3) that there are no time trends in other factors that may affect the serum cholesterol level, we must recognize the possibility of different levels of validity of comparisons within the experiments.

TABLE 10

Experiment J. Serum total cholesterol values for 26 men (groups W, X, Y, Z), while subsisting on "House" and butterfat diets

PERIOD	DIET	DATE	SERUM TOTAL CHOLESTEROL
			mg/100 ml
1	House	2/7–2/14	213.5 ± 6.6[1]
3	Butter	5/11	222.3 ± 6.8
8	Butter	7/19	217.3 ± 6.5
9	House	7/26–8/2	219.0 ± 7.0

[1] Mean and standard error of the mean.

Obviously, in periods 4 and 5, the comparison of corn vs. olive oil diets in groups W and X and of cottonseed oil vs. olive oil in groups Y and Z seem irreproachable and in periods 6 and 7 the comparisons of corn vs. sunflower seed oil and of corn vs. sardine oil are equally free from possible cavil. Comparisons more distant in time, with different intervening diets, require more careful consideration. Table 10 shows that no long-time trends are disclosed when repetitions of the same diet are compared. The mean cholesterol values on the butterfat diet in periods 3 and 8 are very close to each other and those for the "House Diet" in period 1 and period 9 are not significantly different, in spite of the fact that the "House Diet" inevitably is less precisely constant than any

of the other diets. Note that two months between period 2 and period 3 were occupied by subsistence on constant diets but were complicated by pharmacological trials (entirely negative in effect) which accounts for the long interval between the early periods.

The sunflower seed oil diet was tested with groups W and X whereas tests with the cottonseed oil and sardine oil diets were made with groups Y and Z, so the question arises about

TABLE 11

Serum cholesterol differences in experiment J

(Experimental variable approximately 100 gm of fat. Cholesterol difference (mg/100 ml) computed by subtracting the value on the second diet from that on the first. Group WX included 14 men and group YZ 12 men.)

DIET COMPARISON			CHOLESTEROL DIFFERENCES (MG/100 ML) ± S.E.	
			Group WX	Group YZ
Butterfat	*minus*	Olive	+ 35.6 ± 4.7	+ 29.3 ± 4.3
Butterfat	*minus*	Corn	+ 62.8 ± 3.5	+ 59.5 ± 5.7
[1] Olive	*minus*	Corn	+ 27.2 ± 3.6	...
Cottonseed	*minus*	Corn	...	+ 24.0 ± 5.6
[1] Sardine	*minus*	Corn	...	+ 20.1 ± 3.8
[1] Sunflower	*minus*	Corn	+ 9.3 ± 2.8	...
Butterfat	*minus*	House	+ 3.6 ± 3.8	— 0.9 ± 4.4
Butterfat	*minus*	Low-fat	+ 38.8 ± 6.1	...
[1] Olive	*minus*	Sardine	...	+ 10.1 ± 4.6
[1] Olive	*minus*	Cottonseed	...	+ 6.2 ± 5.2

[1] Crossover experiments where half the men were maintained on one diet in parallel with the other half of the men on the comparison diet and the diets were reversed in the subsequent period.

quantitative cross comparisons between these two sets of groups. This is answered by observing the differences in groups W and X vs. Y and Z between the cholesterol values on the butterfat diet (means of periods 3 and 8) and the values on the other diets common to all groups, that is the olive oil and corn oil diets. These comparisons are given in the first two lines of table 11. Groups W, X and Y, Z, exhibited reasonable consistency in their responses to the same dietary differences.

In these comparisons within pairs of groups of men, the corn oil diet is, as in experiment H, consistently associated with the lowest serum cholesterol values, definitely lower than when diets that might be considered as competitors in this respect, namely, olive oil, cottonseed oil, sardine oil and sunflower oil, are used.

TABLE 12

Selected comparisons of changes of serum concentration of total cholesterol and beta lipoprotein cholesterol caused by those substitutions of diet fats which produced the greatest effects. Lipoproteins separated by paper electrophoresis

EXPERIMENT	DIET	NO. OF MEN	CHOLESTEROL	
			Total	Beta
			mg/100 ml	*mg/100 ml*
F	Butterfat	18	245.4 ± 8.5[1]	194.9 ± 11.4
F	Cottonseed	18	203.4 ± 11.3	156.8 ± 8.3
F	Butterfat *minus* cottonseed	18	+ 42.0 ± 4.9	+ 38.1 ± 4.1
HWY	"House"	13	221.3 ± 11.7	177.0 ± 11.7
HWY	Corn	13	160.2 ± 9.0	115.2 ± 8.7
HWY	"House" *minus* corn	13	+ 61.1 ± 5.4	+ 61.8 ± 5.3
HYZ	Coconut	12	224.3 ± 9.0	175.3 ± 14.0
HYZ	"House"	12	213.3 ± 8.2	162.3 ± 10.5
HYZ	Coconut *minus* "House"	12	+ 11.0 ± 2.8	+ 13.0 ± 7.0
JWX	Olive	14	187.9 ± 8.4	130.4 ± 8.4
JWX	Sunflower	14	170.0 ± 9.0	110.2 ± 8.0
JWX	Olive *minus* sunflower	14	+ 17.9 ± 5.1	+ 20.2 ± 4.9

[1] Mean and standard error of the mean.

The diets can be arranged in classes according to their serum cholesterol effects. Butterfat and "House Diets" gave highest serum cholesterol with no significant difference between them. At a lower level of serum cholesterol effect and in order of decreasing serum cholesterol level are the olive oil, low-fat, cottonseed oil and sardine oil diets. The difference between the highest and lowest of these diets is at the borderline of significance. The sunflower oil diet gave a distinctly lower cholesterol level than any of the preceding classes of diets but no direct statistical comparison with the sardine oil diet is possible since the oils were fed to different groups

of men. Finally, the corn oil diet was found in a class by itself yielding the lowest serum cholesterol value of all the diets.

Beta lipoprotein cholesterol changes. Normally, about three-fourths of the total serum cholesterol is found in the beta lipoprotein fraction. In these dietary experiments changes in the beta lipoprotein cholesterol were parallel to those in the total serum cholesterol in every dietary change. As is shown in table 12 the changes in the cholesterol in the beta lipoprotein fraction appear to account for the total cholesterol change.

In every case the change in blood serum cholesterol produced by changing the dietary fat was almost entirely accounted for by the change in the beta lipoprotein fraction.

DISCUSSION

The present results confirm and extend many reports that the fat content of the diet plays a central role in the regulation of the serum cholesterol level in man. It is now well known that a sharp reduction in the fat content of an ordinary American diet generally produces a prompt fall in the serum cholesterol concentration. In America, of course, a low-fat diet means primarily a diet reduced in meat fats and butterfat, since these relatively saturated food fats are most abundant in our ordinary diet. Under these conditions the cholesterol response tends to be more or less directly proportional to the degree of fat restriction so that on diets extremely low in fat, such as the rice-fruit diet, the fall in two or three weeks may amount to 30% or more of the cholesterol level on the unrestricted diet (Keys et al., '50).

The first experiment in the present series (experiment "C," started in 1952), demonstrated the serum cholesterol decrease on a low-fat diet and, further, showed that the isocaloric substitution of 50 gm daily of either olive oil or cottonseed oil for carbohydrate in such a low-fat diet produces an increase in the serum cholesterol concentration. However, this increase did not bring the level up to that characteristic of subsistence

on a "home" diet of the ordinary American type containing about twice as much total fat as that in the olive oil or cottonseed oil diets. Experiment C, then, suggested that olive oil and cottonseed oil are practically the same in their effects and that under these conditions the quantity of total fat in the diet is the major determinant of the serum cholesterol level.

However, experiment F (in 1954) clearly showed that at a level of 100 gm daily in the diet, cottonseed oil is very different from butterfat or the ordinary mixed fats (mainly butterfat and meat fats) in an average American diet. The low-fat diet and the same diet with 100 gm of cottonseed oil substituted for equal calories in the form of carbohydrate were roughly equivalent in regard to serum cholesterol.

Recently other laboratories have been impressed with the low serum cholesterol values resulting from maintenance of patients on formula diets very high in certain vegetable oils, particularly corn oil (Kinsell et al., '52, '53, '54; Ahrens et al., '54; Beveridge et al., '56; Bronte-Stewart et al., '56). Since corn oil is relatively unsaturated and since no such cholesterol-depressing effect is obtained with coconut oil, which is highly saturated, it was natural to propose that the serum cholesterol level is inversely proportional to the amount of unsaturated fat in the diet (Ahrens et al., '54). Further, since linoleic acid glycerides are the main items in the "unsaturated fats" such as corn oil, it has been proposed, without critical analysis, that high serum cholesterol values are expressions of relative deficiency of "essential" fatty acids (Sinclair, '56).

Support was claimed for the essential fatty acid argument by some data indicating that the substitution of oils from fish and marine mammals for the saturated meat and dairy fats in the diet may depress the serum cholesterol level (Bronte-Stewart et al., '56). Marine oils are notably unsaturated, to be sure, but are actually low in the recognized "essential" fatty acids, containing almost no linoleic acid and very little arachidonic acid.

It now appears that much of the effect of corn oil cannot be explained on the basis of these theories about the effects of the fatty acids. We have shown that change from a corn oil diet to sunflower seed oil, which is still more unsaturated and richer in linoleic acid (see table 2), produces a *rise* in serum cholesterol in man and a still greater rise results from changing from corn oil to sardine oil with an iodine number of 188 (Keys, Anderson and Grande, '57). The peculiarity of corn oil is evident in the present results in experiments H and J.

In cholesterol-fed rabbits, corn oil depresses the serum cholesterol more than a "synthetic corn oil" of the same fatty acid composition and crude corn germ has a much greater effect (Jones et al., '56). However, the extrapolation of these results to man is dangerous in view of the obviously great differences between species in cholesterol metabolism. In rats, the effects of different fats on the serum cholesterol level seem to be very different from anything yet suggested about man (Swell et al., '55; Grunbaum et al., '57).

It seems probable that at least three factors are involved in the cholesterol responses to the diets in these several sets of experiments: saturated fatty acids, di-ethenoid fatty acids, and factor "X" in corn germ and crude corn oil. Nothing has been said so far about the mono-ethenoid fatty acids, particularly oleic acid which is perhaps the most abundant of all fatty acids in the foods of the world, and it cannot be guaranteed from present information that these are completely without effect. Finally, it is questionable whether chain length of the fatty acids and the phosphatides in food fats can be ignored entirely.

Comparisons of the present findings with other results reported in the literature on serum cholesterol and the diet in experiments on man is difficult in most cases because of differences in the diet other than in fats, differences in total fat level or lack of detailed specifications as to kinds of fats in the diet. It should be emphasized especially that the present experiments involved diets of ordinary foods prepared by

recipes and arranged in menus that do not depart from the natural eating customs in this country. This is quite different from feeding an emulsion or slurry of a very few ingredients in a synthetic mixture. While the latter are easier to prepare and control, they are not comparable to ordinary "meat-potato-vegetable-fruit-bread" diets. Further, the present experiments cover only the range up to about 40% of calories from total fats. What happens with diets in the unnatural range of 50 to 80% of calories from fats may be very different.

All of the observations in man on this subject have been of relatively short duration and the experiments of the present series are no exception, though they are longer than many from which sweeping conclusions have been drawn. It is conceivable that different results would be obtained from subsistence on these diets over many months or years. In several cases we have maintained experimental diets over periods of 6 months or more and have seen no tendency for the serum cholesterol level to change appreciably after the first few weeks. In Amsterdam, Groen et al. ('52) maintained a dietary experiment for 9 months on human volunteers and observed only a slight progressive tendency for the effects on serum cholesterol to become more marked as time went on after the first few weeks. However, even a slight rate of progression continuing over many years would yield large effects.

The question of possible effects of other nutrients besides fats on the cholesterol level is not involved in the present experiments. Care was taken to maintain constancy of calories and of other nutrients besides the fats so the results pertain only to the fats tested. Perhaps experiment C is an exception in that calorie constancy was not ideal but even in that experiment the dietary variation, other than in fats was not great. Elsewhere we have reported controlled experiments on man in which alterations in protein intake were studied, both on low-fat and on high-fat diets, over the range of around 8 to 20% of total calories from proteins (Keys and Anderson, '57). No effect of the protein level on serum cholesterol was

observed in those experiments and, in any case, the protein intake in the present experiments was constant in the range of about 90 to 100 gm daily.

The carbohydrate intake was constant in amount and kind, within each of the experiments reported here in the comparisons between the various fats. However, the low-fat diets were, necessarily, higher in carbohydrate than the other diets. Considering this point only it would be possible to suggest that the low cholesterol values obtained on the low-fat diets may reflect either the removal of the cholesterol-increasing action of the fats or the addition of a cholesterol-depressing action of carbohydrate. The point may be academic since we are concerned here with the situation in calorie equilibrium and it is not possible to maintain this when the fat level is changed without making a corresponding change in the opposite direction in the carbohydrate intake.

Some of the most striking results in the literature are the cholesterol decreases obtained with corn oil but the diets were of the formula type and the oil was given in extremely large amounts (Kinsell et al., '52, '53, '54). In monkeys, on the other hand, a diet containing 45% of calories as corn oil produced a higher serum cholesterol level than the same diet with 10% of calories as corn oil (Portman, Stare and Bruno, '56).

Mayer et al. ('54) observed *increases* in serum cholesterol in human subjects when 70 gm of peanut oil, corn oil or vegetable oil margarine were added daily for one week to an ordinary American type diet, but no differentiation was made between these vegetable fats. Subsequently they reported a cholesterol *fall* on a mixture of corn oil and vegetable margarine (Beveridge et al., '55). The same group has recently demonstrated a major difference in effect on serum cholesterol between corn oil, on the one hand, and various animal fats on the other, when the experimental fat comprised 60% of the total calories (Beveridge, Connell and Mayer, '56). The greatest cholesterol increasing effect was observed on butterfat and further, they observed that the addition of

corn oil to a fat-free diet produced a decrease in serum cholesterol. None of these results would seem to be at variance with those in the present series, though detailed comparison is not possible.

Hildreth et al. ('51) observed a rise in serum cholesterol in three persons when fats of vegetable origin were added to a low-fat diet but there are no details about the fats used. As in our own earliest experiments (Keys et al., '50), many workers have distinguished only between fats of vegetable and of animal origin in their dietary experiments, so interpretation of the effects of different fats, chemically defined, is difficult or impossible (Groen et al., '52).

SUMMARY AND CONCLUSIONS

1. Total and beta lipoprotein serum cholesterol responses to changes in dietary fats are reported from 4 series of controlled experiments lasting from three to 6 months each, with maintenance for periods of two to 4 weeks in each case on different kinds and amounts of fats. From 16 to 28 men were used in each experiment, the subjects being selected schizophrenics maintained under rigidly standardized conditions in a special metabolic research unit of the Hastings State Hospital. The experiments were designed to eliminate time trends and to cover both directions of change from one fat to another.

2. The diets used conformed to ordinary American foods and menus and were constant and adequate in calories, proteins, vitamins and minerals, the fats being isocalorically substituted for carbohydrates and covering the range of about 9 to 41% of total calories from total fats. The fats studied were: mixed fats in an average American diet both at low-fat (9 to 16% of calories) and high-fat (35 to 41% of calories) levels, olive oil, cottonseed oil, hydrogenated coconut oil, corn oil, sunflower seed oil and sardine oil.

3. The low-fat diets consistently produced a fall in the serum cholesterol concentration from the control (ordinary

U. S. diet) level. Fifty grams daily of olive oil or cottonseed oil introduced into the low-fat diets also produced a significant fall but less marked than the low-fat diet alone.

4. At 50 gm daily of experimental fat, there was no significant difference between olive oil and cottonseed oil in the cholesterol response. Cottonseed oil at 100 gm daily produced a slightly lower cholesterol concentration than did olive oil fed at the same level. The basic low-fat diet also tended to produce a lower cholesterol concentration than 100 gm of olive oil daily. A much more marked depression of serum cholesterol concentration was produced by 100 gm daily of corn oil. Sunflower seed oil and sardine oil also produced significant cholesterol concentration depressions but were less effective than corn oil. Coconut oil, 100 gm daily, produced no significant change in the serum cholesterol compared with the control diet with the same total fat supplied in mixed form. The serum cholesterol values on 100 gm of butterfat daily were somewhat higher than on the control diet equal in total fat content.

5. The changes in the total cholesterol concentration were accounted for, within the limits of error, by the cholesterol in the beta lipoprotein fraction.

6. The serum cholesterol responses to the various fats corresponded roughly to the principle that saturated fats promote higher cholesterol levels than polyunsaturated fats but neither degree of saturation (iodine value) nor content of linoleic acid fully explained the results. Coconut oil is less cholesterol-promoting than would be predicted from the theories that degree of saturation or the content of essential fatty acids is the controlling factor. Sardine oil is considerably less cholesterol-depressing than would be expected if degree of unsaturation is the major factor. And corn oil caused greater depression of serum cholesterol than would be expected from either the essential fatty acid or the degree of unsaturation theories.

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LITERATURE CITED

ABELL, L. L., B. B. LEVY, B. B. BRODIE AND F. E. KENDALL 1952 A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. J. Biol. Chem., *195:* 357.

AHRENS, E. H., JR., D. H. BLANKENHORN AND T. T. TSALTAS 1954 Effect on human serum lipids of substituting plant for animal foods in diet. Proc. Soc. Exp. Biol. Med., *86:* 872.

ANDERSON, J. T., AND A. KEYS 1956 Cholesterol in serum and lipoprotein fractions, its measurement and stability. Clinical Chemistry, *2:* 145.

ANDERSON, J. T., A. LAWLER AND A. KEYS 1957 Weight gain from simple overeating. II. Serum lipids and blood volume. J. Clin. Investigation, *36:* 81.

BEVERIDGE, J. M. R., W. F. CONNELL AND G. A. MAYER 1956 Dietary factors affecting the level of plasma cholesterol in humans: The role of fat. Canad. J. Biochem. and Physiol., *34:* 441.

BEVERIDGE, J. M. R., W. F. CONNELL, G. A. MAYER, J. B. FIRSTBROOK AND M. S. DEWOLFE 1955 The effects of certain vegetable and animal fats on plasma lipids of humans. J. Nutrition, *56:* 311.

BRONTE-STEWART, B., A. ANTONIS, L. EALES AND J. F. BROCK 1956 Effects of feeding different fats on serum-cholesterol level. Lancet, *270:* 521.

GOFMAN, J. W., F. LINDGREN, H. ELLIOTT, W. MANTZ, J. HEWITT, B. STRISOWER, V. HELRING AND T. P. LYON 1950 The role of lipids and lipoproteins in atherosclerosis. Science, *111:* 166.

GROEN, J., B. K. TJIONG, C. E. KAMMINGA AND A. F. WILLEBRANDS 1952 The influence of nutrition, individuality and some other factors including various forms of stress on the serum cholesterol, an experiment of nine months duration in 60 normal human volunteers. Voeding (Amsterdam), *13:* 556.

GRUNBAUM, B., J. R. GEARY, JR., F. GRANDE, J. T. ANDERSON AND D. GLICK 1957 Proc. Soc. Exp. Biol. Med., *94:* 613.

HILDRETH, E. A., S. M. MELLINKOFF, G. W. BLAIR AND D. M. HILDRETH 1951 The effect of vegetable fat ingestion on human serum cholesterol concentration. Circulation, *3:* 641.

JONES, R. J., O. K. REISS AND S. HUFFMAN 1956 Corn oil and hypercholesteremic response in the cholesterol-fed chick. Proc. Soc. Exp. Biol. Med., *93:* 88.

KEYS, A., AND J. T. ANDERSON 1957 Dietary protein and the serum cholesterol level in man. Am. J. Clin. Nutrition, *5:* 29.

KEYS, A., J. T. ANDERSON, F. FIDANZA, M. H. KEYS AND B. SWAHN 1955 Effects of diet on blood lipids in man. Clinical Chemistry, *1:* 34.

KEYS, A., J. T. ANDERSON AND F. GRANDE 1957 "Essential" fatty acids, degree of unsaturation and the effect of corn (Maize) oil on the serum cholesterol in man. Lancet, *272:* 66.

KEYS, A., O. MICKELSEN, E. V. O. MILLER AND C. B. CHAPMAN 1950 The relation in man between cholesterol levels in the diet and in the blood. Science, *112:* 79.

KINSELL, L. W., G. D. MICHAELS, G. C. COCHRANE, J. W. PARTRIDGE, J. P. JAHN AND H. E. BALCH 1954 Effect of vegetable fat on hypercholesterolemia and hyperphospholipidemia. Observations on diabetic and nondiabetic subjects given diets high in vegetable fat and protein. Diabetes, *3:* 113.

KINSELL, L. W., G. MICHAELS, L. DEWIND, J. PARTRIDGE AND L. BOLING 1953 Serum lipids in normal and abnormal subjects. Calif. Med., *78:* 5.

KINSELL, L. W., J. PARTRIDGE, L. BOLING, S. MARGEN AND G. MICHAELS 1952 Dietary modification of serum cholesterol and phospholipid levels. J. Clin. Endocrinology Metab., *12:* 909.

MAYER, G. A., W. F. CONNELL, M. S. DEWOLFE AND J. M. R. BEVERIDGE 1954 Diet and plasma cholesterol levels. Am. J. Clin. Nutrition, *2:* 316.

MELLINKOFF, S. M., T. E. MACHELLA AND J. G. REINHOLD 1950 The effect of a fat-free diet in causing low serum cholesterol. Am. J. Med. Sci., *220:* 203.

PORTMAN, O. W., F. J. STARE AND D. BRUNO 1956 Level and type of dietary fat and experimental hypercholesteremia in the cebus monkey. Fed. Proc., *15:* 570.

SINCLAIR, H. M. 1956 Deficiency of essential fatty acids and atherosclerosis, et cetera. Lancet, *270:* 381.

STARK, H. 1950 Effect of the rice diet on the serum cholesterol fractions of 154 patients with hypertensive vascular disease. Am. J. Med., *9:* 494.

SWELL, L., D. F. FLICK, H. FIELD, JR. AND C. R. TREADWELL 1955 Role of fat and fatty acid in absorption of dietary cholesterol. Am. J. Physiol., *180:* 124.

THE INFLUENCE OF COOKED VS. RAW MAIZE ON THE GROWTH OF RATS RECEIVING A 9% CASEIN RATION [1]

WILLIAM N. PEARSON, SARAH JANE STEMPFEL, J. SALVADOR VALENZUELA, MARGARET H. UTLEY AND WILLIAM J. DARBY

Division of Nutrition, Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee

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INTRODUCTION

Even though pellagra has ceased to be a serious problem in the United States, it is still endemic in certain maize-consuming countries of the world such as Egypt and Yugoslavia. Although the primary cause of pellagra is niacin deficiency the pathogenesis is not simple. For example, cereal diets based largely on rice may contain considerably less niacin than do many maize diets but their consumption does not produce pellagra. Of particular interest is the fact that in Mexico and in the Central American countries where maize is consumed largely in the form of tortilla, there is a low incidence of pellagra. Whether this is due to the coincident ingestion of other foods of relatively high biological value or to some nutritional improvement of the maize during preparation of tortilla has not been definitely established. In the only human feeding trials brought to bear on this problem to date, Goldsmith et al. ('56) reported that women developed niacin deficiency whether whole maize or lime-treated maize was consumed. Unfortunately, in these diets only about 20%

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of the total caloric intake was maize whereas in Central America maize furnishes about 80% of the caloric intake. It is conceivable that if larger amounts of maize were fed a difference between raw and limed maize might have become apparent.

The influence of maize on the niacin requirement of the experimental animal has been the subject of numerous publications. Krehl et al. ('45) found that the addition of 40% maize grits to a diet low in casein depressed the growth of rats. This effect could be overcome by adding niacin or tryptophan to the diet and was attributed to an "amino acid imbalance" and to the effect of maize on the intestinal flora. Laguna and Carpenter ('51) and Cravioto et al. ('52) studied in rats the nutritive value of lime-treated maize and demonstrated that it is superior to that of raw maize. The latter workers suggest that raw maize depresses growth because of an amino acid imbalance but that tortilla does not depress growth because the destruction or racemization of amino acids during processing ameliorates the original imbalance.

The presence of a pellagragenic factor in maize has long been postulated. In recent years the existence of a toxic factor in maize has been suggested by Woolley ('46) and by Borrow et al., ('48). It is conceivable that lime treatment of maize could extract or destroy such a factor but no convincing evidence for this belief has appeared in the literature.

The only postulate regarding the pellagragenic activity of maize for which reasonable experimental evidence exists is that of Kodicek ('40). This worker proposed that maize contains a bound form of niacin that is rendered available to the experimental animal only after hydrolysis with alkali. More recently Kodicek et al. ('56) found that pigs, made niacin deficient on a diet of maize, recovered if the raw maize in the diet was replaced by maize reconstituted from its milling fractions in which the hominy meal and bran had been alkali hydrolyzed.

The present report deals with the effects of cooking and of lime treatment on the nutritive value of maize. It sub-

stantiates the findings of other workers in that lime-treated maize was found to be superior to raw maize as a supplement to a 9% casein diet in the rat. However, data are also presented showing that boiling maize without the addition of lime appreciably enhances its nutritive value. Data concerning amino acid and niacin analyses of the maize products are presented and are related to the current theories regarding the pellagragenic action of maize.

EXPERIMENTAL

Female albino Sprague-Dawley weanling rats were placed singly at random into metal screen-bottom cages, divided into groups of various sizes depending upon the particular experiment, and fed a diet of the following basal composition: sucrose 81.8%; cottonseed oil, 5%; casein, 9%; HMW salts,[2] 4.0%; L-cystine, 0.2%. This was supplemented by the following amounts of vitamins in milligrams per 100 gm of diet: thiamine hydrochloride, 0.2; riboflavin, 0.3; pyridoxine, 0.25; Ca pantothenate, 2.0; choline chloride, 100; inositol, 10; biotin, 0.01; and pteroylglutamic acid, 0.02. Halibut liver oil, fortified with vitamins E and K and diluted with maize oil was added to the daily ration once each week to provide the following daily intakes; vitamin A, 400 I.U.; vitamin D, 4 I.U.; 2-methyl-1, 4-naphthoquinone, 0.06 mg, and alpha-tocopherol, 0.7 mg.

Dietary supplements were given as indicated in table 1. All supplementation of the basal diet was made at the expense of sucrose.

Maize was treated with lime by simmering 2 kg of the whole grain in 4 liters of 1% CaO until the maize was considered soft enough for grinding. This required 3 to 4 hours, a period of time in excess of that used by Cravioto et al. ('45). The mixture was then cooled, adjusted to pH 7.0 with concentrated H_3PO_4, the supernatant liquid decanted, and the

[2] Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14:* 273, 1937.

corn well drained. The exact amount of $Ca_3(PO_4)_2$ appearing in the tortilla and tortilla supernatant was not assessed, but part of the CaO remained undissolved. Therefore, 1% of $Ca_3(PO_4)_2$ was incorporated into the other diets to equate them with respect to these elements.

TABLE 1

Body weight gains and protein efficiency ratios of rats fed a 9% casein diet containing raw maize, boiled maize, or limed maize

SUPPLEMENTS TO BASAL (9% CASEIN) DIET	MEAN DAILY WEIGHT GAIN	PROTEIN EFFICIENCY RATIO	TOTAL NUMBER OF ANIMALS
%	*gm*		
None	1.63	2.00	9
Niacin, 0.003	2.32	2.52	9
Tryptophan, 0.4	2.60	2.56	3
Niacin, 0.003 + tryptophan, 0.4	2.75	2.54	3
Raw maize, 40	2.30	1.86	20
Raw maize, 40 + niacin, 0.003	4.10	2.42	20
Raw maize, 40 + tryptophan, 0.4	3.89	2.19	3
Raw maize, 40 + niacin, 0.003 + tryptophan, 0.4	4.42	2.19	3
Boiled maize, 40	3.25	2.35	9
Boiled maize, 40 + niacin, 0.003	3.56	2.35	9
Boiled maize, 40 + tryptophan, 0.4	3.97	2.40	3
Boiled maize, 40 + niacin, 0.003 + tryptophan, 0.4	3.68	2.06	3
Limed maize, 40	3.60	2.30	9
Limed maize, 40 + niacin, 0.003	3.82	2.36	9
Limed maize, 40 + tryptophan, 0.4	4.07	2.26	3
Limed maize, 40 + niacin, 0.003 + tryptophan, 0.4	3.69	2.09	3
Limed maize, 40 + limed maize ext., 1.2	3.46	2.25	3
Limed maize, 40 + limed maize ext., 1.2 + niacin, 0.003	3.51	2.43	3
Limed maize ext., 1.2	2.40	2.72	3
Limed maize ext., 1.2 + niacin, 0.003	2.63	2.68	3

The wet maize was ground immediately in a meat grinder and spread for drying in shallow glass trays under infra-red lamps and forced air circulation or in a hot air oven at 250°F. This drying process required 12 to 24 hours. The dried product was ground to a fine powder for incorporation into the ex-

perimental diets. The collected supernatant was similarly dried and ground to a powder and is referred to as "dried" extract in table 1.

Boiled maize was prepared in the same manner as lime-treated maize except that it was simmered for 3 to 4 hours in tap water. After cooling, a neutral mixture of CaO and H_3PO_4 was added. The wet maize was dried and ground as indicated for the lime-treated maize.

The niacin and tryptophan contents of raw maize, boiled maize, limed maize, and limed-maize extract, were determined microbiologically using *L. arabinosus* as the assay organism. For "total" niacin assay 0.5 gm of each sample was hydrolyzed for 30 minutes with 1 N H_2SO_4. Water extracts of the maize products were prepared by autoclaving 0.5 gm in distilled water for 30 minutes or by extracting it by stirring for half an hour in a large volume of water at room temperature. The methods used in the paper chromatographic studies of niacin will be described in conjunction with the results of the studies. Samples for assay of "total" tryptophan were hydrolyzed according to the method of Greene and Black ('44) and extracts were prepared for assay of "free" tryptophan by hot water extraction as noted above. Assays were read either turbidimetrically or titrimetrically depending on the particular experiment. The two methods of measurement gave comparable results.

Commercially prepared assay media were employed for the determination of leucine, methionine, lysine, isoleucine, cystine, and phenylalanine using *Leuconostoc mesenteroides* ATCC 18042 as the test organism. *Streptococcus faecalis* 29–21, and *Lactobacillus arabinosus* ATCC 8014 were used for the assay of threonine and histidine respectively according to the procedure of Stokes et al. ('45).

The maize samples were prepared for amino acid assay as follows: ± 0.5 gm of sample was accurately weighed and placed into a small ampule with 5 ml of 10% HCl. The ampule was then evacuated by use of a water pump and sealed in a flame. After autoclaving for 16 hours at 15 lbs. pressure

the extract was cooled, filtered and adjusted to pH 6.8 for assay. Cystine assays were carried out on the alkaline hydrolysates prepared for tryptophan assays. Assays for "free" amino acids were carried out on the hot water extracts previously described.

RESULTS AND DISCUSSION

Rat growth studies. Growth data for 30-day periods are presented (table 1) as mean daily weight gains and protein efficiency ratios. These values were derived from as many as 4 separate experiments, but not all groups were represented in each experiment. The figures within parentheses indicate the number of animals from which the means are derived.

Before discussing the differences between dietary groups it is appropriate to note the variability of response of animals fed a diet supplemented with 40% raw maize. Most animals on this diet grew poorly, but an occasional animal grew rather well. In one experiment to determine if the animals which were most retarded in growth were niacin deficient, two were supplemented with 3 mg % of niacin at the completion of the 30-day experimental period. Upon the introduction of niacin into the diet the negligible growth was accelerated at an unusually rapid rate, indicating that these rats were truly niacin deficient. In other experiments, not presented, it has been found that depleting animals for one week on a protein-free, niacin-free diet did not reduce this variability, suggesting that different niacin or tryptophan reserves were not responsible. The reason for this variability is not apparent. However, there are numerous possible explanations, such as differences in the niacin requirement of individual animals; in the rate of synthesis of niacin in the intestine or in the rate of conversion of tryptophan to niacin or in the ability to utilize "bound" niacin.

When 40% limed maize is added to a niacin-free, 9% casein diet growth is superior to that obtained with raw maize or boiled maize. Boiled maize permits better growth than raw maize, but in our studies, although the limed maize permits

somewhat better growth, both boiled maize and limed maize have identical protein efficiency ratios. This indicates that boiling *per se* is sufficient to enhance the biological value of maize and that the apparent role of lime has been overemphasized through failure to employ cooked rather than raw maize as the standard of reference.

A dried extract of limed maize was fed in combination with limed maize in one experiment. There was no difference in the growth obtained when compared to that of limed maize alone. When the 9% basal diet was supplemented with this extract growth was somewhat enhanced. This was probably due to the niacin and tryptophan supplied by the extract. This evidence fails to support the hypothesis of a "toxic factor" in the limed maize. The results could be interpreted to indicate that if any toxic factor is present it is destroyed rather than extracted.

Niacin supplementation of the basal diet and of that containing raw maize enhanced both growth and protein efficiency ratios. Niacin supplementation of boiled or limed maize diets did not appreciably improve growth or protein efficiency ratios. Tryptophan supplementation enhances growth on all diets, but again protein-efficiency values were increased only on the basal and raw maize diets. In general, supplementation with both niacin and tryptophan did not improve growth or protein efficiency ratios above those obtained on either nutrient alone.

Chromatographic studies. The poor growth obtained with raw maize can be readily corrected with niacin or tryptophan whereas these nutrients do not appreciably improve the growth obtained with limed maize or boiled maize. For this reason it appeared as if niacin, tryptophan or both of these nutrients, might be more available from the latter products. Accordingly, microbiological analyses for niacin and tryptophan were performed.

Table 2 details the partition of total, water extractable, and non-water extractable niacin and tryptophan in the various dietary supplements studied. The total tryptophan content

of the three samples was essentially the same. Practically no free tryptophan was present even in the cooked maize indicating that hydrolysis did not take place. Under our conditions of preparation raw maize contains nearly twice as much total niacin as does limed maize and one third again as much niacin as does boiled maize. However, practically all of the niacin in limed and boiled maize is readily extractable by hot water whereas less than 50% of the raw-maize niacin is extracted by such a procedure. Similar values were found when 10 gm samples were extracted at room temperature by stirring for one-half hour with one liter of distilled water. Because of its mildness the latter method was adopted for the remainder of the study.

TABLE 2

Niacin and tryptophan contents of maize supplements

	TOTAL NIACIN A	WATER EXTRACTABLE NIACIN B	NON-WATER EXTRACTABLE NIACIN (A–B)	TOTAL TRYPTOPHAN C	"FREE" TRYPTOPHAN D	PROTEIN-BOUND TRYPTOPHAN (C–D)
	μg/gm	*μg/gm*	*μg/gm*	*μg/gm*	*μg/gm*	*μg/gm*
Raw maize	27.5	12.8	14.7	675.0	25.1	649.1
Boiled maize	17.7	16.6	1.1	641.9	15.3	626.6
Limed maize	15.3	13.4	1.9	671.9	14.9	657.0

In order to determine the predominant form of niacin present in these water extracts they were chromatographed using the ascending technique on Whatman no. 1 filter paper in *n*-butanol saturated with water. After a development period of 12 to 16 hours the chromatograms were dried and assayed for niacin activity using *Lactobacillus arabinosus* as the test organism. This assay was carried out by placing consecutive 0.5 inch sections of the chromatograms into individual tubes. Then either 10 ml of assay medium was added, followed by sterilization in the autoclave and inoculation or 10 ml of sterile inoculated medium was added directly. In the latter case, the paper was not subjected to the rigors of autoclaving. The assays were read turbidimetrically.

It is evident from figure 1 that the water extractable niacin activity present in boiled and limed maize occurs exclusively as niacin but that a small part of the niacin activity of raw maize is present in a form that does not move from the origin of the chromatogram.

After these initial analyses had been completed the publication of Kodicek et al. ('56) came to our attention in which chromatographic evidence was presented indicating that maize

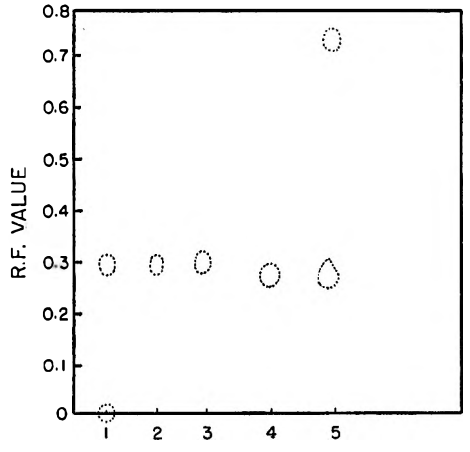

Fig. 1 Paper chromatograms of room temperature water extracts of maize, limed maize, and boiled maize. Solvent: *n*-butanol saturated with water. Areas of microbiological activity are enclosed by the broken lines. 1, raw maize; 2, limed maize; 3, boiled maize; 4, niacin; 5, niacin + niacinamide.

contained no free niacin. On this account it was deemed advisable to repeat our own work and attempt to duplicate that of Kodicek.

Accordingly, 5 gm of ground maize was refluxed for one hour on a water bath with 20 ml of 80% (v/v) methanol that was 0.1 N in HCl. After centrifugation and removal of the supernatant the residue was re-extracted once with 20 ml and twice with 10 ml of the same solvent. The combined extracts were then evaporated to half their volume (30 ml) on a steam bath. Instead of using ion-exchange resin at this point to remove HCl as was done by Kodicek et al., the evaporation

was continued at 40°C. and 25 to 30 mm pressure until small volume (± 5 ml) was reached. Fifty milliliters of distilled water were added and the evaporation was again carried out. This procedure was repeated to make a total of three extractions. The final dry residue was taken up in 1 ml of distilled water for chromatography.

The water extract of maize was prepared by stirring 10 gm of maize in one liter of distilled water for 30 minutes. After filtration the extract was evaporated to a convenient small volume (1 to 2 ml) at 40°C. and 25 to 30 mm pressure. For determination of the "free" and "bound" niacin of Kodicek et al., suitable aliquots of these extracts were incubated with 1 N NaOH at room temperature for 30 minutes and then neutralized with 1 N HCl. These hydrolyzed and non-hydrolyzed extracts were chromatographed with and without the addition of authentic niacin and niacinamide in *n*-butanol saturated with water. The chromatograms were developed for 12 to 16 hours using the ascending technique. Niacin-active areas were detected by use of the cyanogen bromide method of Kodicek and Reddi ('51) and by microbiological assay using *Lactobacillus arabinosus* as the test organism. The microbiological determinations were carried out by placing consecutive 0.5 inch sections of the chromatogram into individual tubes containing 10 ml of inoculated assay medium. This technique has the advantage over conventional plate bioautography in that quantitative data are obtained as well as rf values.

The results of this experiment are detailed in figure 2. It can be seen that niacin could not be detected in the unhydrolyzed methanol-HCl extract of maize (fig. 2 A, 1 and 2) by either method of assay. In this particular experiment the "bound" niacin detected at the chromatogram origin by the CNBr reaction failed to support growth of *L. arabinosus*. However, as will be seen presently, this was not always the case and either the bound form is slightly active *per se* or else some liberation of free niacin occurs under the conditions of assay.

After alkaline hydrolysis (fig. 2 A, 3 and 4) the areas of microbiological activity coincided with the CNBr reactive areas. When authentic niacin was added to this mixture before chromatography no additional spots were detected. A mixture of niacin and niacinamide (fig. 2 A, 5) also gave coincident results. These findings, then, confirm those of Kodicek et al. ('56) and, in addition, demonstrate the absence

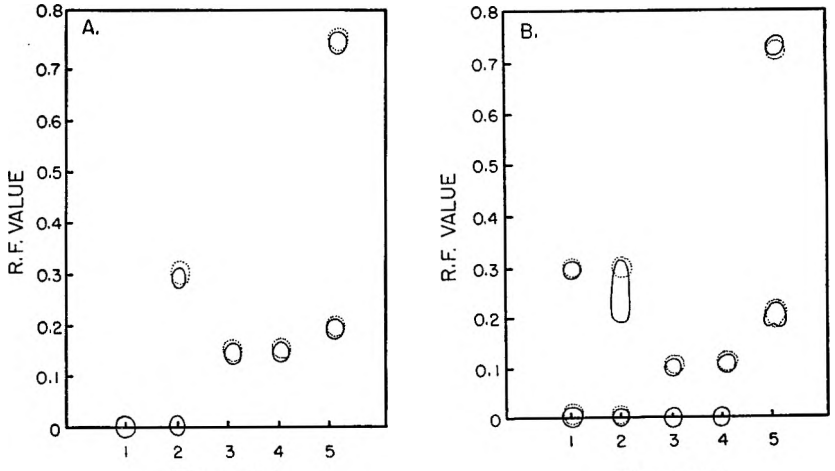

Figure 2 A Figure 2 B

Fig. 2 Paper chromatograms of (A) 80% methanol-HCl extract of maize and (B) water extract of maize. Solvent: *n*-butanol saturated with water. Areas giving the cyanogen-bromide reaction are enclosed by solid lines. Areas of microbiological activity are enclosed by the broken lines. 1, unhydrolyzed extract; 2, unhydrolyzed extract + niacin; 3, alkali-hydrolyzed extract; 4, alkali hydrolyzed extract + niacin; 5, niacin + niacinamide, 1 μg each.

of microbiologically available niacin in a methanol-HCl extract of maize.

The chromatographic patterns obtained using a water extract of maize contrast sharply with those obtained with the methanol-HCl extract. Figure 2 B (samples 1 and 2) shows that free niacin was detected by both the chemical and microbiological techniques. In addition, "bound" niacin was also present in the water extract and exhibited slight microbiologi-

cal activity. Hydrolysis of the water extract with NaOH and subsequent neutralization resulted in a marked change in the niacin rf value and a loss of microbial activity at the chromatogram origin. However, a complete coincident loss of CNBr activity at the origin did not take place, suggesting that this method of detection may not be as specific as is the microbiological technique.

Further studies were made to quantitate more accurately the partition of niacin and its "bound" form in water extracts of raw maize. In one study, water extracts of maize made at room temperature were chromatographed and the chromatograms were cut up and assayed for niacin content in the manner previously described. The two forms of niacin were actually separated and then determined. Since the "total" niacin content of such extracts (H_2SO_4 hydrolysis) had been determined previously, the "bound" niacin could be estimated indirectly by subtracting the free niacin content (as measured on the chromatogram) or directly by subjecting the origin of the chromatogram to alkaline hydrolysis prior to assay. Both methods gave similar values.

The other technique employed was to assay directly the water extracts of maize with and without prior hydrolysis. Alkaline and, in some cases, acid hydrolysates were prepared by autoclaving the water extracts with 1 N NaOH or H_2SO_4 for 30 minutes at 15 lbs. pressure and neutralizing for assay. In each of these procedures the microbiological assays were made both aseptically and non-aseptically. In the latter case, the "bound" niacin in the non-hydrolyzed extracts was not subjected to the hydrolytic action of autoclaving. The aseptic assays were titrated after 72 hours of incubation but, to lessen the risk of contamination, the non-aseptic assays were read turbidimetrically after 24 hours. The results of these studies are shown in tables 3 and 4.

It can be seen in table 3 that autoclaving the chromatographic strips with the assay medium (pH 6.8 to 7.0) is sufficient to break down the "bound" niacin in an almost quantitative manner and thus render it microbiologically

TABLE 3

Niacin partition in water extracts of maize determined by microbiological assay of paper chromatograms

SAMPLE CHROMATOGRAPHED	NIACIN CONTENT μG/0.5 ML EXTRACT							
	Method of assay	"Bound"	Rf	Free	Rf	% Bound	Predicted total content (Tube assay)	% (Recovery total)
(1) H_2O Extract	Aseptic	0.201	0	0.298	0.32	...	0.515	104[1]
(2) H_2O Extract: NaOH treated	Aseptic	0.242	0	0.263	0.33	48	0.515	98
(3) H_2O Extract	Non-aseptic	0.030	0	0.343	0.44	...	0.515	97
(4) H_2O Extract: NaOH treated	Non-aseptic	0.157	0	0.374	0.37	29.6	0.515	103

[1] Total recovery calculated using "bound" niacin content obtained on NaOH-treated extract, and the predicted total content determined by tube assay of an NaOH-treated extract.

TABLE 4

Niacin partition in water extracts of maize as determined by non-aseptic microbiological assay

SAMPLE ASSAYED	NIACIN CONTENT			
	(A) No treatment	(B) Hydrolysis[1] with NaOH or H_2SO_4	(B-A) Bound niacin	Bound niacin
	μg/gm	*μg/gm*	*μg/gm*	*%*
H_2O extract of maize: ½ hour at room temperature	6.76	18.36	11.60	63.2
H_2O extract of maize: 10 hours at room temperature	14.22	20.33	6.11	26.2
H_2O extract of maize: Soxhlet extracted for 15 hours	28.62	29.07	0.45	1.5

[1] The value for the acid and alkaline hydrolyzate were so similar that they are presented here as an average.

available. The possibility also exists that *L. arabinosus* can use the bound form over a 72-hour incubation period. If the non-aseptic technique is employed, virtually no activity can be detected at the origin demonstrating that *L. arabinosus* cannot use the bound form if its growth period is limited to 24 hours.

Both aseptic and non-aseptic assays were performed on chromatograms of the same water extract but at different times. In spite of the fact that the sample was stored in the frozen state between determinations it can be seen that there is a substantial decrease in the amount of "bound" niacin in samples 3 and 4 as compared to samples 1 and 2, accompanied by a concomintant increase in "free" niacin. The lability of the "bound" form on standing in aqueous solution is also suggested in table 4. In this instance, the sample was extracted and assayed immediately so that there was no storage effect. This resulted in the finding that 63.2% of the niacin present occurred as the "bound" form — the highest figure obtained in this laboratory using aqueous extracts. This lability in aqueous solution of the "bound" form may account for the finding of Laguna and Carpenter ('51) that merely wetting maize diets before feeding is sufficient to improve the growth of rats.

The various forms of niacin were quantitatively extracted from maize and converted almost entirely to microbiologically available form by Soxhlet extraction with water (table 4). Under such circumstances only 1.5% of the niacin could be classified as "bound" and this is well within the experimental error of the method. This contrasts with the finding that somewhat more than one-half of the niacin is water extracted at room temperature by stirring for one-half hour. Increasing the extraction time to 10 hours at room temperature increases but slightly the amount of niacin extracted but does yield a higher percentage of free niacin. This again is a reflection of the lability of the bound form in aqueous solution.

The most logical explanation for the lack of quantitative niacin extraction is that the starchier components of the grain are easily extracted whereas the more fibrous portions of the grain and the oily germ are not. Since exhaustive Soxhlet extraction removes the niacin quantitatively it is probable that the partition of niacin in the fraction that is not extracted at room temperature is identical with that that is.

These results demonstrate that the apparent forms of niacin present in maize vary according to the method of extraction. Although our results indicate that "bound" niacin represents from 30 to 60% of the niacin activity of maize, it is probable that only "bound" niacin is present in maize — this being readily converted to the free form in aqueous solution. Additional evidence for this belief is that only "bound" niacin can be detected in methanol · HCl extracts. The rapid conversion of the "bound" to the free form in water explains our finding that boiled maize is as effective as limed maize in supporting the growth of rats. In essence, our findings lend considerable support to the claim of Kodicek et al. that the effectiveness of lime treatment of maize in curing a niacin deficiency depends upon the degree of hydrolysis of the "bound" form of niacin.

Amino acid analyses. Massieu et al. ('49) reported that considerable loss of amino acids occurred during the preparation of tortilla and Cravioto and co-workers ('52) suggested that this might result in an improved amino acid balance. Since imbalances of certain amino acids have been demonstrated to produce niacin deficiency in rats fed low-protein diets (Koeppe and Henderson, '55) it was considered pertinent to study this possibility.

The tryptophan loss during the cooking process proved to vary considerably from batch to batch (tables 2 and 5). The very low tryptophan content of maize contributes to this variability in that relatively small quantitative differences assume large proportions when calculated on a percentage basis. Consequently, we attach no particular significance to this lack of agreement and feel that the superiority of cooked

maize cannot be explained on the basis of alterations in the amount or availability of tryptophan.

Further amino acid analyses of our maize products are presented in table 5 along with the percentage losses calculated from these data and those of Massieu et al. Considering

TABLE 5

The amino acid content of raw maize, boiled maize and limed maize [1,2]

AMINO ACID	TOTAL CONTENT						% LOSS FROM LIMED MAIZE	
	Raw maize		Boiled maize		Limed maize		This study	Calculated from Massieu ('49)
	mg/gm dry wt.		*mg/gm dry wt.*		*mg/gm dry wt.*			
	Total	Free	Total	Free [3]	Total	Free [3]		
Arginine	7.6	0.10	6.2	<0.007	6.2	<0.020	18.4	16
Leucine	9.4	0.12	9.6	<0.009	9.5	<0.001	0	4
Lysine	3.1	0.14	2.0	<0.007	1.9	<0.006	36.8	12
Methionine	2.5	0.05	2.6	....	2.5	....	0	10
Cystine	1.1	0.11	0.7	....	0.7	....	36	..
Phenylalanine	3.9	0.07	3.8	0	3.8	0	0	0
Isoleucine	7.5	0.10	7.0	0	7.2	0	5	..
Histidine	2.5	0.14	2.0	....	2.1	....	18	26
Valine	5.1	0.14	4.5	....	4.6	....	11	0
Tryptophan	1.1	0.03	0.8	<0.002	0.9	<0.003	22	33
Threonine	2.7	0.13	2.4	<0.005	2.5	<0.002	9.4	25
Mean moisture content	12.4%		6.0%		4.4%			

[1] After hydrolysis with 10% HCl for 16 hours at 15 lbs. pressure, except in the cases of tryptophan and cystine, which were run on alkaline hydrolyzates.

[2] Three separate batches of each product were analyzed.

[3] It was impossible to assay the free amino acid content of these maize products with precision because of their very low potency.

the differences in preparation, our results agree fairly well with those of this group except in the cases of tryptophan, lysine, and threonine. Tryptophan has been discussed previously. The large loss of lysine found in our laboratory is undoubtedly due to the more severe heat treatment during our preparations resulting in a more severe "browning" reaction.

The large loss of threonine reported by the Mexican workers could not be confirmed in our study. However, because of the findings of Koeppe and Henderson ('55) that the addition of threonine to a low-tryptophan diet can produce a niacin deficiency in the rat, a study was made of the effects of the addition of threonine to the cooked maize diets. The results indicated that threonine was without effect which dispelled the possibility that a cooking loss of threonine might have effectively lowered the niacin requirement. It might be added that a similar study of lysine supplementation of the cooked products was also without effect. Very little hydrolysis, if any, took place during the cooking process; practically no free amino acids could be detected in the cooked products. Presumably, the small measurable quantities found in the raw maize are lost in the cooking water.

The fact that no gross changes in amino acid content were observed plus the finding that supplementation with the two amino acids deemed most likely to influence the niacin requirement was ineffectual, detracts considerably from the "amino acid imbalance concept" proposed by Cravioto et al. ('52). Although reduced digestibility of the cooked product with the preferential excretion of a grossly imbalanced protein fraction (such as zein) could conceivably improve the balance in the intact animal, satisfactory experimental evidence has not been obtained for this belief. Consequently, it is concluded that the superiority of cooked maize to raw maize in the 9% casein diet of the rat is due to the liberation of free niacin from the normally unavailable "bound" form during the cooking process. The chemical nature of this "bound" niacin has not been determined.

SUMMARY

Data have been presented confirming reports in the literature that limed maize permits more rapid growth of rats than does raw maize when added to a 9% casein, niacin-free diet. The finding that boiled maize was equivalent in this

respect indicates that heat treatment alone increases the supplemental value of maize.

Amino acid analyses of raw maize, limed maize, and boiled maize failed to reveal striking differences in composition. In addition, those amino acids deemed most likely to increase the niacin requirement by producing an amino acid imbalance (lysine and threonine) did not alter growth when added to the cooked maize diets. From these data it is concluded that the cooked maize effect cannot be explained on the basis of an improved amino acid balance.

Chromatographic evidence was obtained for the presence of a "bound" form of niacin in raw maize. This "bound" form can be extracted with acidic 80% methanol and is quite stable in this solvent. The "bound" niacin is also readily extracted by distilled water at room temperature but is relatively unstable under such conditions, yielding free niacin on standing. Both limed and boiled maize were found by paper chromatography to contain only free niacin, whereas raw maize contained either 100% "bound" niacin, in the case of acidic methanol extraction, or 30 to 60% "bound" niacin by water extraction. The lability of the "bound" form in aqueous solution suggests that the niacin present in maize occurs largely as the "bound" form as maintained by Kodicek et al. ('56). The conversion of this to free niacin by cooking in either limed water or water alone would appear to explain the superiority of these products in the diet of the rat.

The apparent unavailability of the "bound" form of niacin to the rat and pig (Kodicek et al., '56) suggests that this form may also be unavailable to the human. If so, this finding may explain, in part, the low incidence of pellagra in tortilla-consuming populations. However, the lability of the bound form in aqueous solution would tend to militate against this possibility.

LITERATURE CITED

BORROW, A., L. FOWDEN, M. M. STEDMAN, J. C. WATERLOW AND R. A. WEBB 1948 A growth-retarding factor in maize bran. Lancet, *254:* 752.

CRAVIOTO, R. O., R. K. ANDERSON, E. E. LOCKHART, F. P. MIRANDA AND R. S. HARRIS 1945 Nutritive value of the Mexican tortilla. Science, *102:* 91.

CRAVIOTO, R. O., G. H. MASSIEU, O. Y. CRAVIOTO AND F. DE M. FIGUEROA 1952 Effect of untreated corn and Mexican tortilla upon the growth of rats on a niacin-tryptophan deficient diet. J. Nutrition, *48:* 453.

GOLDSMITH, G. A., J. GIBBENS, W. G. UNGLAUB AND O. N. MILLER 1956 Studies of the niacin requirement in man. III. Comparative effects of diets containing lime-treated and untreated corn in the production of experimental pellagra. Am. J. Clin. Nutrition, *4:* 151.

GREENE, R. D., AND A. BLACK 1943 A microbiological method for the determination of tryptophane. Proc. Soc. Exp. Biol. Med., *54:* 322.

KODICEK, E. 1940 Estimation of nicotinic acid in animal tissues, blood and certain foodstuffs. I. Method. Biochem. J., *34:* 712.

KODICEK, E., AND K. K. REDDI 1951 Paper chromatography of nicotinic acid derivatives. Nature, *168:* 475.

KODICEK, E., R. BRAUDE, S. K. KON AND K. G. MITCHELL 1956 The effect of alkaline hydrolysis of maize on the availability of its nicotinic acid to the pig. Brit. J. Nutrition, *10:* 51.

KOEPPE, O. J., AND L. M. HENDERSON 1955 Niacin-tryptophan deficiency resulting from imbalances in amino acid diets. J. Nutrition, *55:* 23.

KREHL, W. A., L. J. TEPLY AND C. A. ELVEHJEM 1945 Corn as an etiological factor in the production of a nicotinic acid deficiency in the rat. Science, *101:* 283.

LAGUNA, J., AND K. J. CARPENTER 1951 Raw vs. processed corn in niacin deficient diets. J. Nutrition, *45:* 21.

MASSIEU, G. H., J. GUZMAN, R. O. CRAVIOTO AND J. CALVO 1949 Determination of some essential amino acids in several uncooked and cooked Mexican foodstuffs. Ibid., *38:* 293.

STOKES, J. L., M. GUNNESS, M. DWYER AND M. CASWELL 1945 Microbiological methods for the determination of amino acids. J. Biol. Chem., *160:* 35.

WOOLLEY, D. W. 1946 Occurrence of a pellagragenic agent in corn. Ibid., *163:* 773.

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