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February 1960

VOLUME 70
NUMBER 2

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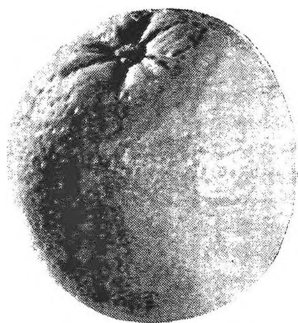

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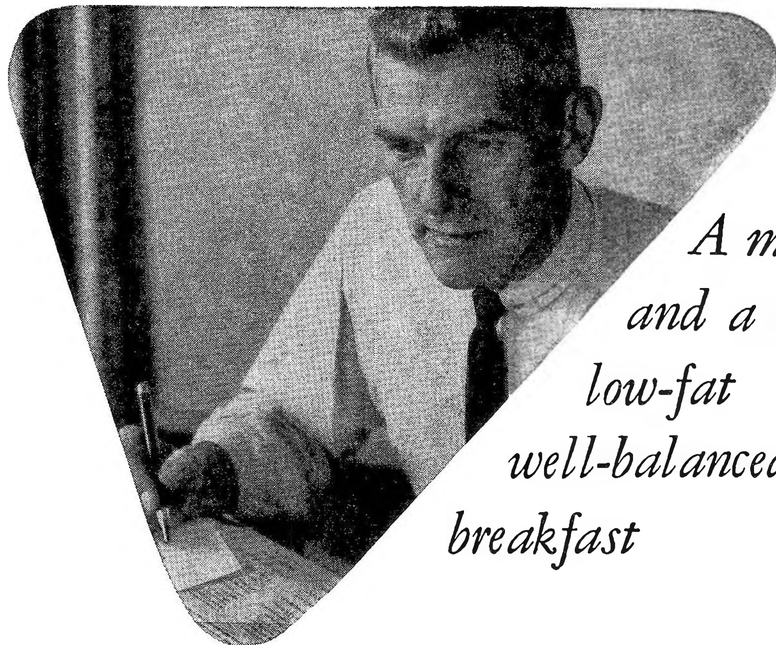

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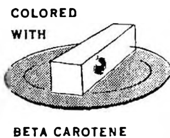

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Arginine Requirement of the Chick and the Arginine-Sparing Value of Related Compounds[1]

J. E. SAVAGE AND B. L. O'DELL
Departments of Poultry Husbandry and Agricultural Chemistry, University of Missouri, Columbia

The arginine requirement of the growing chicken and the relation of this requirement to other dietary constituents has been investigated extensively in recent years (Almquist and Merritt, '50; Wietlake et al., '54; Monson et al., '55; Snyder et al., '56; Fisher et al., '56; Hogan et al., '57; Krautmann et al., '58; Edwards et al., '58; Scott and Forbes, '58). There are wide discrepancies in the quantitative requirements reported but this is to be expected in view of the marked variations in basal diets. It is well known that the amino acid requirement for growth of an animal depends in part on the age of the animal, the protein, energy and amino acid composition of the diet and the availability of the amino acid under study. Determination of the arginine requirement of the chick is further complicated by other dietary components.

Almquist et al. ('41) found that creatine, creatinine and guanidoacetic acid stimulated growth of chicks fed an arginine-deficient diet. This fact has since been confirmed by numerous investigators but little quantitative data is available concerning their sparing effect. Hegsted et al. ('41) pointed out the significance of glycine in chick nutrition. This amino acid is commonly accepted as a dietary essential for the growing chick, but its function in metabolism and relationship to the arginine requirement is not entirely clear.

It was the purpose of this investigation to determine the arginine requirement of the chick fed high-protein, high-energy diets with and without added glycine, to determine the quantitative arginine-sparing value of creatine and guanidoacetic acid and to study the effect of high levels of glycine on the arginine requirement.

EXPERIMENTAL

Single Comb White Leghorn cockerels hatched from the University flock were used. At hatching they were banded and groups of 10 placed on wire-mesh floors in electrically heated batteries. Feed and water were supplied ad libitum. The chicks were weighed weekly for 4 weeks and then rated for leg paralysis and degree of feathering.

The basal diet used was slightly modified from that described by Wietlake et al. ('54). Its percentage composition was: casein, 35; glucose hydrate, 45.3; cellulose,[2] 3.0; salts, modified to supply 60 ppm of zinc, 5.0; $CaCO_3$, 1.0; soybean oil, 10.0; DL-methionine, 0.5; choline Cl, 0.2; antioxidant,[3] 0.0125. The vitamin supplement was the same as described except that it supplied 0.04 mg of biotin per 100 gm of diet. The casein was analyzed for arginine by the microbiological method previously described (O'Dell et al., '58), and on the basis of 16% of nitrogen in the casein, contained 3.8%. The basal ration contained 31.5% of crude protein and thus 1.2% of arginine. When supplements of glycine, arginine hydrochloride, creatine hydrate, creatinine and guanidoacetic acid were included, they were substituted for an equivalent amount of glucose.

Received for publication September 5, 1959.

[1] Contribution from the Missouri Agricultural Experiment Station, Journal Series no. 2062. Approved by the Director. A preliminary account of this investigation has been published (Savage and O'Dell, '56).

[2] Solka Floc, a wood pulp, obtained from Brown Company, Chicago.

[3] Santoquin (1,2 - dihydro - 6 - ethoxy - 2,2,4 - trimethyl-quinoline), obtained from Monsanto Chemical Company, St. Louis.

RESULTS

In the first series of trials, graded levels of arginine were added to the basal diet and to a similar diet supplemented with 1.5% of glycine. The growth responses are shown graphically in figure 1 in which the 4-week weights are plotted against the logarithm of the calculated arginine content of the diets. The horizontal lines represent what is considered to be maximal weight under the conditions imposed and the ascending lines were calculated by the method of least squares. The equation for the curve representing response with no added glycine is $Y = 837X + 72$ and with 1.5% of added glycine it is $Y = 1082X + 53$, where Y is the weight in grams at 4 weeks and X is the logarithm of the percentage of arginine in the diet.

Glycine improved the rate of growth and feather development at all levels of arginine and had an ameliorative effect on leg weakness. The beneficial effect on growth was magnified with increasing levels of arginine. At an arginine concentration of 2.4%, which is more than enough to support maximal growth under these conditions, the chicks that received glycine weighed 38 grams more, a difference that is statistically significant at the 1% level.

The point of intersection of the ascending and horizontal lines for the groups that received no supplementary glycine occurred at 2.25% of arginine, and that for the glycine supplemented groups at 2.10%. If one assumes that these points represent the minimal arginine requirement for the type of diet used, it is clear that, in addition to stimulating growth in its own right, glycine spared the requirement for arginine.

The data that relate to the arginine sparing value of various supplements are shown in table 1. All diets contained 1.5%

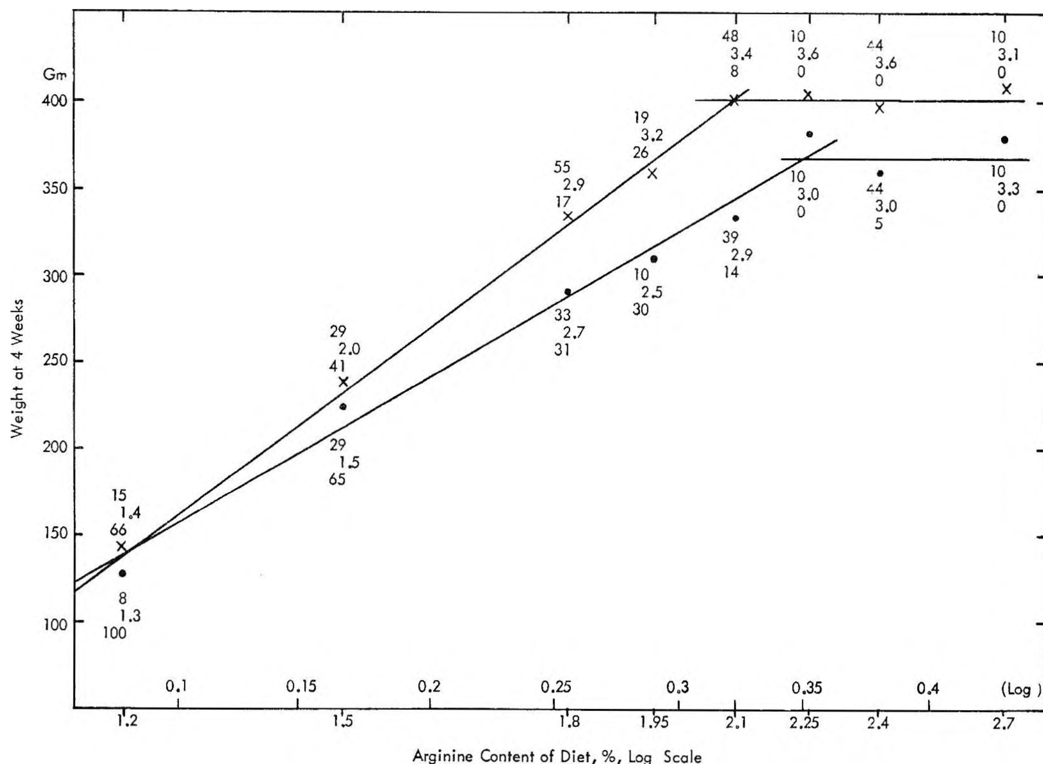

Fig. 1 Arginine requirement of chicks fed casein with and without glycine supplementation. Line X = 1.5% of added glycine, line ● = no added glycine. The numbers below the data points in descending order are: number of chicks represented, feather score and percentage of leg paralysis.

of glycine and arginine was fed at three different levels. The average weights at 4 weeks, feather score and percentage of leg paralysis are presented along with the calculated percentage of arginine which was spared. The latter value was calculated from the upper curve in figure 1 by subtracting the percentage of arginine in the basal diet from the percentage which correlated with the weight observed. For example, when 2.0% of creatine hydrate was added to the basal diet (A, line 3), the average weight was 281 gm which corresponds to 1.62% of arginine. The basal diet contained 1.2%, and thus, 0.42% of arginine was spared.

The sparing action of creatine was not in direct proportion to the level fed although the amount spared varied somewhat with the level of arginine in the diet. About 1% of creatine was required to give the maximal response when the basal ration contained 1.2% of arginine. Under these conditions, approximately 0.4% of arginine was spared by creatine and about 0.2% by guanidoacetic acid. The latter compound was toxic when fed at the 1.6% level. In trials not reported here, higher levels of methionine (0.8%) and choline (0.4%) did not improve the response to guanidoacetic acid.

When the basal ration contained 1.5% of arginine the maximal response was obtained with 0.5% of creatine and about 0.3% of arginine was spared. In the presence of 1.8% of arginine the maximal response was obtained with 0.3% of creatine, a level which spared about 0.25% of

TABLE 1

Arginine-sparing value of creatine, guanidoacetic acid and creatinine

Supplement		Number of chicks	Weight at 4 weeks	Feather score[1]	Leg paralysis[2]	Arginine spared[3]
Name	Amount					
	%		gm		%	%
A. Basal ration (1.5% glycine; no arginine added)						
None	—	15	142 ± 10[4]	1.4	66	—
Creatine	4.0	7	277 ± 28	1.7	29	0.40
Creatine	2.0	20	281 ± 25	2.5	30	0.42
Creatine	1.5	18	262 ± 26	2.5	50	0.36
Creatine	1.0	9	268 ± 35	2.0	33	0.38
Creatine	0.5	8	215 ± 40	1.9	75	0.21
Guanidoacetic acid	1.6	9	167 ± 24	2.4	56	0.07
Guanidoacetic acid	0.8	17	217 ± 12	1.9	47	0.21
Guanidoacetic acid	0.4	18	220 ± 24	1.7	66	0.23
B. 1.5% glycine; 0.3% arginine added						
None	—	29	238 ± 12	2.0	41	—
Creatine	1.0	20	333 ± 22	3.0	25	0.31
Creatine	0.5	9	335 ± 34	3.0	11	0.32
Guanidoacetic acid	0.8	10	340 ± 28	3.7	10	0.34
Guanidoacetic acid	0.4	10	261 ± 38	2.4	40	0.06
C. 1.5% glycine; 0.6% arginine added						
None	—	68	320 ± 12	2.9	34	—
Creatine	1.00	49	386 ± 9	3.5	4	0.24
Creatine	0.50	30	409 ± 12	3.3	13	0.30
Creatine	0.30	40	387 ± 13	3.4	18	0.24
Creatine	0.15	48	374 ± 13	3.3	25	0.19
Creatine	0.10	18	342 ± 21	3.1	39	0.05
Creatine	0.05	30	338 ± 19	3.0	33	0.04
Guanidoacetic acid	0.8	19	335 ± 17	3.1	21	0.03
Guanidoacetic acid	0.4	18	358 ± 17	3.1	6	0.12
Guanidoacetic acid	0.2	20	346 ± 19	2.9	45	0.06
Creatinine	0.5	30	378 ± 14	3.3	20	0.20

[1] Poorest wing feathers were scored 1; perfect feathers scored 4 and others received the respective intermediate values.

[2] Chicks that showed a stilted gait, "spraddle-leg" or paralysis at 4 weeks.

[3] Calculated from the curve shown in figure 1.

[4] Standard error of the mean.

TABLE 2

Relative arginine-sparing values of glycine and creatine

Ration no.	Ration supplements			Chicks at 4 weeks	
	Arginine	Glycine	Creatine hydrate	No.	Weight
	%	%	%		gm
5148	0.6	1.5	—	68	339 ± 12
5163	0.6	3.0	—	67	393 ± 10
5360	0.6	4.5	—	30	409 ± 11
5359	0.6	1.5	1.0	47	415 ± 10
5164	0.6	3.0	1.0	59	425 ± 7
5423	1.2	—	—	18	397 ± 13
5151	1.2	1.5	—	60	417 ± 6
5166	1.2	3.0	—	58	418 ± 6
5362	1.2	4.5	—	29	426 ± 9
5424	1.2	—	1.0	18	429 ± 10
5165	1.2	1.5	1.0	45	431 ± 10
5167	1.2	3.0	1.0	47	425 ± 6

Significance of differences by *t* test

Rations compared	*t*	P	Rations compared	*t*	P
5163 vs. 5148	3.42	< 0.01	5362 vs. 5151	0.83	> 0.05
5360 vs. 5163	0.95	> 0.05	5424 vs. 5423	1.98	$= 0.05$
5164 vs. 5359	0.80	> 0.05	5362 vs. 5423	1.89	*ca.* 0.05

arginine. It is interesting to note that 0.15% of creatine spared 0.19% of arginine. Under these conditions, guanidoacetic acid was much less effective than creatine, whereas 0.5% of creatinine spared nearly as much arginine as the higher levels of creatine. Although creatine exerts a sparing effect when arginine is suboptimal, there is little or no evidence that it stimulates growth when the diet contains adequate arginine. (Compare ration no. 5166 and 5167 in table 2.)

The effect of adding what might be termed excessive levels of glycine to the diets that contained two levels of arginine, with and without added creatine, is shown in table 2. Although glycine is commonly used in purified diets at levels of 1.0 to 1.5%, it is clear that higher levels stimulated the rate of gain when the basal diet contained suboptimal arginine. For example, compare ration no. 5148 and 5163 which contained 1.8% of arginine. The growth rate of animals fed 3.0% of glycine was significantly greater than that of those fed 1.5%. Although the rate using 4.5% was slightly better than that at 3.0% it was not statistically significant In the presence of 1.0% of creatine, 3.0% of glycine supported only slightly more rapid gain than 1.5%. These trials were run at a later date than those described in figure 1, and the maximal rate of gain was somewhat greater.

When the basal diet contained 2.4% of arginine, supplementation with 1.5% of glycine (compare ration no. 5151 and 5423) gave the expected response, but higher levels of glycine had no additional effect as it did when the diet contained only 1.8% arginine. Thus, it seems clear that at a suboptimal level of arginine, glycine has true sparing action for arginine. Although creatine did not improve growth when added to diets that contained adequate arginine and glycine (compare ration no. 5167 and 5166), it produced a significant response when added to a diet that contained 2.4% of arginine and no added glycine, (compare ration no. 5424 and 5423). In fact, the diet with 1% of creatine and no added glycine (ration no. 5424) supported the maximal rate of gain. One might say that creatine spared the glycine requirement since the addition of glycine did not stimulate further growth.

DISCUSSION

Various suggestions have been offered to explain the apparently higher arginine

requirement of chicks fed diets based on casein, supplemented with free arginine compared with those fed a practical corn-soya diet. Krautman et al. ('58) postulated an unrecognized factor in corn and soybean-oil meal that spares arginine. Edwards et al. ('58) found that betaine, choline and methionine, as well as creatine and guanidoacetic acid, stimulated chick growth on diets low in arginine. They suggested that practical diets may contain these arginine-sparing compounds. The results of the present study confirm their findings that creatine and its precursors spare arginine. Glycine is a common constituent of protein and its concentration in the diet has an important effect on the arginine requirement. Little is known about the concentration of the other precursors in natural feedstuffs.

The possibility that the arginine in casein is not digestible was ruled out by the observations of O'Dell et al. ('58). These investigators also found a greater than normal concentration of urea in the urine of chicks fed supplementary arginine. This observation led to the postulation that when free arginine is fed, it is absorbed rapidly and a higher than normal proportion is degraded by kidney arginase. Hence, an apparently higher requirement results when the free amino acid is fed.

Fisher et al. ('55) found, in contrast to previous observations, that chicks can tolerate glycine in excess of 4% and indeed that such high levels improved feed utilization. The beneficial effect of glycine levels as high as 4.5% has been demonstrated here when the diet is suboptimal in arginine. At least part of its benefit must be related to creatine biosynthesis but the possibility that it inhibits catabolism of arginine should not be overlooked.

It seems unlikely that the chick converts any appreciable quantity of creatine to arginine. Presumably it spares arginine because the performed creatine eliminates the need for arginine in the biosynthesis of creatine. Creatine at a level of 0.3% was able to spare about an equal percentage of arginine, and higher levels did not spare more. If the amidine group were enzymatically used to form arginine one would expect more conversion with massive doses. It is noteworthy that creatinine, which is considered rather inert in mammals, exerts a marked arginine sparing action in the chick.

SUMMARY

Male White Leghorn chicks were reared to 4 weeks of age on a basal, purified diet that contained 35% of casein and 10% of soybean oil. The diet, which contained by analysis 1.2% of arginine, was supplemented with 0.5% of methionine and in some cases with glycine. Graded levels of arginine, creatine, guanidoacetic acid and glycine were added as supplements.

Under these conditions, the arginine requirement for maximal growth without added glycine was 2.25% and with 1.5% of added glycine it was 2.1% of the diet.

When the basal diet contained 1.2% of arginine, creatine spared about 0.4% of arginine and 1% creatine was as effective as higher levels. When the basal diet contained 1.5% of arginine, about 0.3% of arginine was spared by 0.5% of creatine, and when it contained 1.8% of arginine, 0.25% was spared by 0.3% of creatine. Creatinine spared slightly less arginine than creatine, and guanidoacetic acid spared about one-half as much.

When the arginine level was suboptimal, 3.0% glycine supported a faster rate of gain than 1.5% but not when adequate arginine or arginine plus creatine were present. When sufficient arginine and insufficient glycine were present, creatine improved the growth rate and thus may be said to spare the glycine requirement.

[4] The authors gratefully acknowledge the materials given by the following: Allied Chemical Corporation for creatine and arginine; Dow Chemical Company for methionine and glycine; Monsanto Chemical Company for glycine, guanidoacetic acid and Santoquin; General Mills, Inc. for arginine; Hoffman-La Roche, Inc. for biotin; American Cyanamid Company for folic acid; Distillation Products Industries for vitamin A, and Merck, Sharp and Dohme for the other vitamins used.

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Amino Acid Requirement for Maintenance in the Adult Rooster

III. THE REQUIREMENTS FOR LEUCINE, ISOLEUCINE, VALINE AND THREONINE, WITH REFERENCE ALSO TO THE UTILIZATION OF THE D-ISOMERS OF VALINE, THREONINE AND ISOLEUCINE[1]

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The maintenance requirements for leucine, isoleucine, valine, and threonine have been determined in young men (Rose et al., '55a, b, c) and women (Leverton et al., '56a, b, c; Swendseid and Dunn, '56) and the adult rat (Benditt et al., '50; Nasset et al., '51; Womack et al., '53; Burroughs et al., '40). Such requirements have not been determined, however, for the adult rooster.

Studies on the utilization of the optical isomers of threonine and isoleucine have shown only the natural or L-form to be active for the adult human (Rose et al., '55a, b), the growing chick (Grau and Peterson, '46; Grau, '49), mouse (Bauer and Berg, '43) and rat (Rose, '38). Rose ('38) concluded that the D-isomer of leucine was not available for growth in the rat or for the maintenance of nitrogen equilibrium in the adult human (Rose et al., '55b); Bauer and Berg ('43) also reported that D-leucine failed to support growth in the mouse.

The D-form of leucine has been reported available for growth in the chick (Grau and Peterson, '46). Similar findings have been reported for the growing rat by Rechcigl et al., ('58a) who also showed that the utilization of D-leucine is reduced in the presence of norleucine (Rechcigl et al., '58b).

The unnatural form, D-valine, has been reported to be unavailable for growth in the chick (Grau and Peterson, '46), mouse (Bauer and Berg, '43) and rat (Rose et al., '55c). White et al. ('52) reported, however, that the D-isomer of valine possessed approximately one-half the growth promoting properties of the L-isomer for the young rat. This discrepancy was resolved by Wretlind ('56), who showed that D-leucine interferred with the utilization of D-valine but that D-valine, when the natural form of leucine is supplied, promotes growth in the rat.

In the present report, data are presented on the requirements for leucine, isoleucine, valine and threonine for the maintenance of nitrogen equilibrium in the adult rooster not depleted of protein reserves. Information is also presented on the utilization of the D-isomers of valine, threonine, and isoleucine.

EXPERIMENTAL

White Leghorn roosters, at least 12 months old, were used in all studies. The animals were maintained and standardized prior to receiving the test diets as previously described (Leveille and Fisher, '58).[2]

In all experiments the diets were fed in a pelleted form and the animals were trained to consume their daily allotment of

Received for publication July 22, 1959.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, New Brunswick. Supported in part by grants-in-aid from the National Science Foundation and the Grange League Federation.

[2] We would like to thank the following for materials supplied: Merck, Sharp and Dohme, Rahway, New Jersey; Warner-Chilcott Laboratories, Morris Plains, New York and Distillation Products Industries, Rochester.

26 gm/kg within a single 30-minute period.

The composition of the complete starting diet was identical to diet B previously described by Leveille and Fisher ('59). The purity of the amino acids used as well as the specific rotation of the optical isomers was 100 ± 1% of theoretical.

The experimental procedure used has also been described previously (Leveille and Fisher, '59). The length of the feeding period was increased from 5 to 6 days for technical reasons and nitrogen balance was determined during the last three days of each period. The findings to be reported should not necessarily be extended beyond the experimental feeding period herein employed.

RESULTS

Results are presented in tables 1–4. In a previous report (Leveille and Fisher, '59) it was pointed out that if nitrogen intake remained constant, nitrogen balance was merely a reflection of nitrogen excretion, and emphasis was placed on excretion rather than on balance data. In the present studies, presumably because of amino acid imbalances induced by the lower levels of certain amino acids, the animals did not consume all the diet presented to them and therefore nitrogen intake varied. For this reason nitrogen balance is emphasized in the present report.

The previously described definitions (Leveille and Fisher, '59) of adequacy, the maintenance requirement and minimal level, still remain valid and form the basis of quantitation. As previously described, the *maintenance requirement* was taken as the lowest level of an amino acid that would maintain the same degree of nitrogen balance as that observed on the complete starting diet; and the *minimal maintenance level* was taken as the lowest level of an amino acid that would maintain nitrogen equilibrium. The values satisfying these definitions were estimated by fitting nitrogen balance against milligrams of amino acid/26 gm of diet by the method of least squares. Through the use of fitted curves every observation is included in the estimation of requirement values. The equations showing the smallest residual variance were linear for some amino acids and curvilinear for others. The intersection of the calculated curve with a line drawn at the level of nitrogen balance obtained on the complete starting diet was taken as the maintenance requirement. The intersection of the least square line with the line of zero balance was taken as the minimal maintenance level. The standard error of estimate for the calculated line was arbitrarily used to obtain a range for the maintenance requirement.

As mentioned above, nitrogen balance was plotted against milligram of amino acid/26 gm of diet. This value is identical to the intake of the amino acid when all of the diet fed is consumed (26 gm/kg). The use of the quantity of amino acid in 26 gm of diet, rather than the amount ingested is justified by the fact that consumption was depressed only at the two lowest levels fed with all the amino acids studied (tables 1–4).

The body weight changes observed were in all cases less than 5%; the smaller changes could have been due to changes in water balance and feather losses and the greater weight changes were usually observed at the lower levels of the amino acids fed, in conjunction with food refusal. The larger weight losses were undoubtedly partly due to the amino acid deficiency but probably resulted largely from the reduced caloric intake.

From the data presented in table 1 it is evident that as the level of leucine was decreased there was a concomitant decrease in nitrogen balance. When these data are calculated to obtain the best-fitting least squares equation a curvilinear relationship is obtained ($Y = 1.573X - 0.005X^2 - 70$) from which the maintenance requirement is estimated as 124 mg/kg day of L-leucine and the minimal maintenance level as 54 mg/kg/day. If the range of the requirement is taken as ± the standard error of estimate from the calculated curve (± 17 mg/kg/day), the range of the maintenance requirement is 83 to 166 mg/kg/day.

The data relating to the isoleucine requirement (table 2) show that the nitrogen balance decreased as the level of isoleucine decreased. The maintenance requirement as estimated from the least

TABLE 1
Maintenance requirement for L-leucine in the adult rooster

L-Leucine/ 26 gm diet	Body weight			Nitrogen			Diet consumed
	Initial	Final	Δ	Intake	Excretion	Balance	
mg	*gm*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*%*
166 (4)[1]	2530	2580	+50	281	233 ± 8[2]	+48	100
83 (4)	2580	2622	+42	276	237 ± 10	+39	100
42 (4)	2622	2620	−2	274	263 ± 10	+11	100
21 (4)	2620	2585	−35	174	245 ± 8	−71	61
0 (4)	2585	2478	−107	136	185 ± 10	−49	50

[1] Number of animals indicated within parentheses.
[2] Mean ± standard error.

TABLE 2
Maintenance requirement for isoleucine in the adult rooster

Isoleucine/ 26 gm diet	Body weight			Nitrogen			Diet consumed
	Initial	Final	Δ	Intake	Excretion	Balance	
mg	*gm*	*gm*	*gm*	*mg/kg/day*	*mg/kg/day*	*mg/kg/day*	*%*
DL 265 (4)[1]	2436	2451	+51	278	250 ± 18[2]	+28	100
DL 133 (4)	2451	2476	+25	276	252 ± 8	+24	100
DL 68 (4)	2476	2474	−2	275	277 ± 8	−2	100
DL 35 (4)	2474	2458	−16	241	311 ± 9	−70	90
0 (4)	2458	2331	−127	131	230 ± 15	−99	44
L 49 (4)	2562	2571	+9	283	272 ± 18	+11	92

[1] Number of animals indicated within parentheses.
[2] Mean ± standard error.

squares equation ($Y = 1.189X - 0.003X^2 - 88$) is 144 mg/kg/day with a range of 124 to 165 mg/kg/day when the standard error of estimate is considered (± 7 mg/kg/day). The minimal maintenance level is approximately 98 mg/kg/day. These requirements are for the racemic mixture of the amino acid; when the natural isomer of isoleucine was incorporated into the diet exclusively at a level of 49 mg/26 gm of diet, a nitrogen balance of 11 mg/kg/day resulted (table 2). This balance is equivalent to that obtained with the level of 120 mg/26 gm of diet for the racemic mixture, indicating that the D-isomer is apparently not utilized by the adult rooster. The requirements, therefore, expressed as milligram/kilogram/day of the L-isomer of isoleucine, are taken to be 72 and 49 for the maintenance requirement and minimal maintenance level, respectively.

The data presented in table 3 demonstrate the sharp decrease in nitrogen balance observed with the first decrease in the level of threonine from that in the complete starting diet. From these data the maintenance requirement for DL-threonine is taken as the highest level fed, 148 mg/kg/day; the minimal maintenance level is considered to be 110 mg/kg/day. The range for the maintenance requirement as calculated from the standard error (± 7 mg) of the regression equation ($Y = 0.718X - 79$) is 147–165 mg DL-threonine/kg/day. From table 3 it can be seen that L-threoine, when fed at a level of 73 mg/26 gm of diet, resulted in a positive balance of 15 mg/kg/day, which is equivalent to 132 mg of DL-threonine/26 gm of diet; or the L-isomer is approximately twice as effective as the racemic mixture. These data indicate that the D-isomer of threonine is also not available to the adult rooster and further support the requirement determined with DL-threonine. The requirements thus are taken as 74 and 55 mg/kg/day of L-threonine for the maintenance requirement and minimal maintenance level, respectively.

TABLE 3

Maintenance requirement for threonine in the adult rooster

Threonine/ 26 gm diet	Body weight			Nitrogen			Diet consumed
	Initial	Final	Δ	Intake	Excretion	Balance	
mg	*gm*	*gm*	*gm*	*mg/kg/ day*	*mg/kg/ day*	*mg/kg/ day*	*%*
DL 148 (3)[1]	2743	2473	0	280	245 ± 12[2]	+35	100
DL 73 (3)	2473	2452	−21	282	332 ± 19	−50	100
DL 36 (3)	2452	2422	−30	285	323 ± 7	−38	100
DL 18 (3)	2422	2357	−65	270	330 ± 13	−60	93
0 (3)	2357	2278	−79	230	313 ± 20	−83	76
L 73 (4)	2379	2384	+5	281	266 ± 6	+15	100

[1] Number of animals indicated within parentheses.
[2] Mean ± standard error.

TABLE 4

Maintenance requirement for valine in the adult rooster

Valine/ 26 gm diet	Body weight			Nitrogen			Diet consumed
	Initial	Final	Δ	Intake	Excretion	Balance	
mg	*gm*	*gm*	*gm*	*mg/kg/ day*	*mg/kg/ day*	*mg/kg/ day*	*%*
DL 234 (3)[1]	2480	2463	−17	284	267 ± 5[2]	+17	100
DL 125 (3)	2463	2472	+9	285	263 ± 13	+22	100
DL 62 (3)	2472	2472	+0	284	268 ± 8	+16	100
DL 31 (3)	2742	2423	−49	283	350 ± 12	−67	100
0 (3)	2423	2315	−108	207	394 ± 49	−187	70
L 31 (3)	2510	2742	−38	266	345 ± 12	−79	94

[1] Number of animals indicated within parentheses.
[2] Mean ± standard error.

The data presented for valine (table 4) show that the nitrogen balance remained unchanged on the first three levels fed and then decreased sharply at the next two lower levels. The maintenance requirement estimated from a least squares line through the three lowest levels fed ($Y = 3.274X - 181$) is 61 mg/kg/day, and the minimal maintenance level 55 mg/kg/day expressed as DL-valine. The requirements expressed as the natural isomer, however, remain unchanged, since D-valine is apparently completely utilized by the adult rooster. A level of 31 mg of L-valine/26 gm of diet resulted in a nitrogen balance of −79 mg/kg/day (table 4), which is very similar to the balance obtained with the same quantity of DL-valine, suggesting an equal utilization for the D and L isomers.

DISCUSSION

The results observed demonstrate the essentiality of leucine, isoleucine, valine and threonine for the maintenance of nitrogen equilibrium in the adult rooster. This is in accord with observations in other species. However, the requirements differ quantitatively from those of the rat (Benditt et al., '50) and the adult human (Rose et al., '55a, b, c; Leverton et al., '56a, b, c). Such variations are not surprising in view of the differences in the composition of the tissues being synthesized.

In a previous report (Leveille and Fisher, '59), it was proposed that the requirements of the adult animal are largely determined by the composition of the tissue being synthesized—in the case of the rooster, feather synthesis. It was also shown that arginine, histidine and lysine fit this hypothesis. The results of the present study for threonine, leucine and isoleucine are essentially in accord with this hypothesis, although the requirement for valine is lower than would be anticipated from the feather content of this amino acid. The validity of this concept

must await the further determination of the maintenance requirements for the remaining amino acids, methionine, cystine, phenylalanine, tyrosine and tryptophan. From the evidence obtained thus far, it appears that although feather synthesis is not the only factor influencing the maintenance requirement of the adult rooster, it is probably a very important one.

The refusal of the animals to consume all of the diet presented is indicative of an amino acid imbalance (Harper and Kumpta, '59). The fact that the 4 amino acids studied are so intimately related to each other with reference to amino acid balance (Harper, '58) may have influenced the requirements observed. If lower levels of these 4 amino acids were fed simultaneously, lower requirements might possibly have been observed. This appears unlikely, however, since the refusal to consume all of the diet was encountered only when the amino acid levels had been lowered to the point where the animals had reached the region of negative nitrogen balance. Therefore, it is unlikely that an imbalance existed at amino acid levels greater than those which caused food refusal.

The observed food refusal might be attributed to a deficiency of an essential amino acid *per se* as contrasted with an amino acid imbalance. Unpublished data obtained in this laboratory show that adult roosters receiving a diet devoid of sulfur-containing amino acids will consume all of the food presented, and animals receiving such a diet are in negative nitrogen balance. This observation does not support the concept that an essential amino acid deficiency *per se* will cause a decrease in food consumption under the conditions of the present investigations. That an amino acid imbalance will depress food intake has been clearly demonstrated by Harper ('59), who showed that the feeding of an imbalanced amino acid diet to rats would depress food intake within three hours.

The results, which indicate that the D-isomers of threonine and isoleucine are not available to the adult rooster, are in accord with those of other workers in other species (Rose et al., '55b, c; Bauer and Berg, '43) and for the growing chick (Grau and Peterson, '46; Grau, '49). The present observations on the availability of D-valine are in accord with the studies of White et al. ('52) and Wretlind ('56). The unavailability of the D-isomer observed by Rose ('38) in the rat and Bauer and Berg ('43) in the mouse has been shown to be due to the presence of the D-isomer of leucine. Grau and Peterson ('46) reported that D-valine was not utilized by the growing chick; a closer inspection of their data, however, shows that when D-valine was fed alone, a utilization of 38% resulted when compared with the L-isomer, and when the racemic mixture was fed a utilization of 33% was obtained for D-valine. To the writers' knowledge the present report is the first to indicate complete utilization of D-valine. It should be kept in mind, however, that this conclusion is based only on data obtained with 4 animals during a 6-day feeding period. It is conceivable that the adult rooster, with a much lower requirement and presumably a better-developed metabolic system, could utilize D-valine to a greater extent than the growing chick.

SUMMARY

The maintenance requirements for leucine, isoleucine, valine and threonine have been determined in the adult rooster not depleted of his protein reserves. Using a 6-day feeding period, nitrogen balance was employed as a criterion of adequacy. The ability of the adult rooster to utilize the D-isomers of valine, threonine and isoleucine was also determined.

The maintenance requirement for L-leucine was estimated to be 124 mg/kg/day and the minimal maintenance level, 54 mg/kg/day.

The maintenance requirement for L-isoleucine was found to be approximately 72 mg/kg/day, and the minimal maintenance level 49 mg/kg/day. The maintenance requirement for L-threonine was 74 mg/kg/day, and the minimal maintenance level, 55 mg/kg/day.

The maintenance requirement for L- or DL-valine was taken to be 61 mg/kg/day, and the minimal maintenance level 55 mg/kg/day.

Comparative studies with the L- and DL-isomers suggested that the D-isomers

of threonine and isoleucine were unavailable while D-valine appeared to be completely available for the maintenance of nitrogen equilibrium in the adult rooster.

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Amino Acid Balance and Imbalance

III. QUANTITATIVE STUDIES OF IMBALANCES IN DIETS CONTAINING FIBRIN[1]

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The causes of adverse effects that have been observed in experimental animals as a result of dietary additions of amino acids have been classified as amino acid imbalances, antagonisms and toxicities (Harper, '58). Imbalances can be created in two ways. The most consistent and reproducible manner is by adding a fairly large amount (3 to 20% of the diet) of an unbalanced protein, or an amino acid mixture lacking one indispensable amino acid, to a diet that is low in protein (Harper, '58, '59; Salmon, '58). Occasionally, however, a small supplement (0.2 to 1.0% of the diet) of one or two amino acids has been found to cause an imbalance that is quite severe (Hankes et al., '49; Deshpande et al., '58a; '58b). As the adverse effects are, in both cases, prevented completely by supplementing the diet with the indispensable amino acid that is in shortest supply, and, as this is the criterion by which an amino acid imbalance is distinguished from an antagonism or a toxic effect, both of these conditions are classed as imbalances. The question arises, however, whether imbalances produced in these two ways differ in other respects. The observation that an imbalance, resulting in depressions in growth rate, food consumption and nitrogen retention, occurs when small quantities of methionine and phenylalanine are added to diets containing 6% of fibrin as the source of protein (Deshpande et al., '58a, '58b; Kumta et al., '58; Harper and Kumta, '59), provided an opportunity to study imbalances of both types using a single basal diet.

Since amino acid imbalances, induced by including mixtures of amino acids lacking a single amino acid, can be demonstrated over a range of protein levels (Harper, '59), whereas the imbalance caused by adding a small amount of threonine to a diet lacking niacin and containing only 9% of casein cannot be demonstrated if the casein level is slightly increased (Henderson et al., '53), the effect of the level of dietary protein on imbalances produced by both methods in diets containing fibrin has been studied. Also, since few quantitative studies have been made of the influence of amino acid imbalances on amino acid requirements, growth responses of rats fed imbalanced diets to which graded levels of the limiting amino acid were added have been measured.

EXPERIMENTAL

Male weanling rats of either the Sprague Dawley or the Holtzman strain, weighing 40 to 50 gm, were used. They were housed in individual suspended cages and fed the basal diet (described below) ad libitum for a preliminary period of 4 to 5 days to allow them to adapt to the new environment. Rats that showed a progressive weight gain were selected and separated into groups of 5 which did not differ in average initial weight by more than one gram. These groups of rats were then offered the various experimental diets ad libitum and weighed at least twice weekly during the two-week experimental period.

The basal diet used was essentially that described earlier (Kumta et al., '58). This contained fibrin, 6; salt mixture, 5 (Harper, '59); corn oil, 5; vitamin supplements, 0.25 (Harper, '59); choline chlor-

Received for publication July 6, 1959.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Nutrition Foundation, New York.

ide, 0.15 and dextrin as the carbohydrate to make up 100%.[2] All diets were kept refrigerated. Changes in protein level and additions of amino acids as indicated in the tables of results, were compensated for by adjusting the percentage of dextrin.

RESULTS

The effects of adding graded levels of DL-methionine and DL-phenylalanine to the basal diet are shown in table 1. The rate of growth decreased as the levels of methionine and phenylalanine were increased from 0.05 and 0.075% to 0.2 and 0.3%, respectively, but no greater growth retardation occurred when the levels of DL-methionine and DL-phenylalanine were raised to 0.6 and 0.9%, respectively.

The effects of different levels of leucine, isoleucine, valine and histidine in overcoming the imbalance produced by 0.2% of DL-methionine and 0.3% of DL-phenylalanine are shown in table 1. Although the gain in weight of rats fed the diet containing methionine and phenylalanine was depressed by about 40%, only small amounts of leucine, isoleucine, valine and histidine were needed to overcome the effect of the imbalance. Increasing the levels of these 4 amino acids above the minimal level tested had no further growth promoting effect. As these 4 amino acids are equally limiting in fibrin, all must be added together to overcome the effects of the imbalance (Deshpande et al., '58a).

The results of experiments designed to study the effect of an imbalance in increasing the need for each of these amino acids individually are presented in figure 1. When an amino acid mixture lacking in histidine was fed (A), a considerable depression in growth rate occurred. The rats in this group lost weight initially and at the end of 14 days had gained only 2 gm. The growth rate of the group fed this diet supplemented with only 0.05% of L-histidine approached that of the control group fed the basal diet containing only the fibrin. The growth response was still greater when 0.1% of L-histidine was added. These results show the extent to which the level of the limiting amino acid, histidine, had to be increased just to prevent the imbalance caused by the mixture of amino acids lacking histidine.

Effects of amino acid mixtures lacking valine, isoleucine and leucine are shown in figures 1-B, 1-C and 1-D respectively. In each case the level of the limiting amino acid needed to overcome the growth retardation caused by the imbalance can be estimated closely from the curves.

As the food intake of rats ingesting an imbalanced diet falls off, the intake of the limiting amino acid must also drop.[3]

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

[3] ——— 1959 Amino acid balance and total food consumption. Nutrition Rev., 17: 122.

TABLE 1

Effect of including various amino acid supplements in diets containing 6% of fibrin

Diet	Amino acid supplements						Gain in weight
	DL-methionine	DL-phenylalanine	L-leucine	DL-isoleucine	DL-valine	L-histidine	
	%	%	%	%	%	%	*gm/2 weeks*
1	—	—	—	—	—	—	37.4 ± 1.9[1]
2	0.05	0.075	—	—	—	—	28.8 ± 2.3
3	0.1	0.15	—	—	—	—	24.6 ± 3.2
4	0.2	0.3	—	—	—	—	21.3 ± 2.6
5	0.4	0.6	—	—	—	—	19.8 ± 2.6
6	0.6	0.9	—	—	—	—	20.0 ± 2.1
7	—	—	—	—	—	—	32.2 ± 3.4
8	0.2	0.3	—	—	—	—	19.8 ± 2.9
9	0.2	0.3	0.1	0.1	0.15	0.05	45.6 ± 2.3
10	0.2	0.3	0.2	0.2	0.3	0.1	47.2 ± 2.6
11	0.2	0.3	0.3	0.3	0.45	0.15	48.2 ± 3.5
12	0.2	0.3	0.4	0.4	0.6	0.2	49.8 ± 3.1

[1] Standard error of the mean for 5 rats.

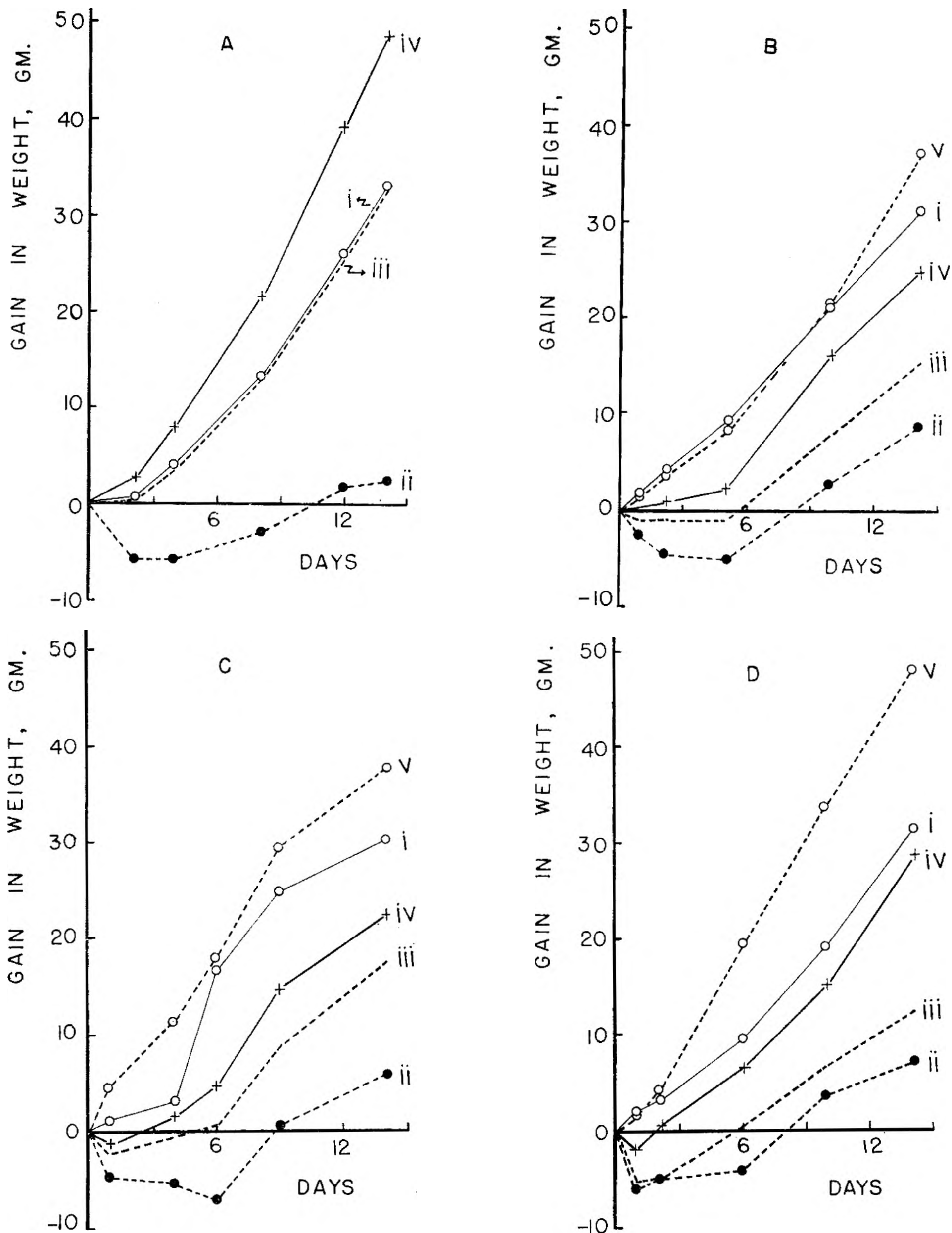

Fig. 1 Effect of amino acid mixtures* lacking single amino acids on the increased need for the limiting acid(s).

A, (i) Basal diet (6% fibrin); (ii) basal diet + amino acid mixture lacking histidine; (iii) as for (ii) + 0.05% L-histidine; (iv) as for (ii) + 0.1% L-histidine.

B, (i) Basal diet (6% fibrin); (ii) basal diet + amino acid mixture lacking valine; (iii) as for (ii) + 0.05% DL-valine; (iv) as for (ii) + 0.1% DL-valine; (v) as for (ii) + 0.2% DL-valine.

C, (i) Basal diet (6% fibrin); (ii) basal diet + amino acid mixture lacking isoleucine; (iii) as for (ii) + 0.05% DL-isoleucine; (iv) as for (ii) + 0.1% DL-isoleucine; (v) as for (ii) + 0.2% DL-isoleucine.

D, (i) Basal diet (6% fibrin); (ii) basal diet + amino acid mixture lacking leucine; (iii) as for (ii) + 0.05% L-leucine; (iv) as for (ii) + 0.1% L-leucine; (v) as for (ii) + 0.2% L-leucine.

* The amino acid mixture provided as percentage of the diet: DL-methionine, 0.4; DL-phenylalanine, 0.6; L-leucine, 0.4; DL-isoleucine, 0.4; DL-valine, 0.7; L-lysine·HCl, 0.6; L-arginine·HCl, 0.2; L-tryptophan, 0.2; DL-threonine, 0.4; L-glutamic acid, 1.0; and L-histidine·HCl, 0.4.

Therefore, the food intake of several of the groups used in the previous study were measured. The results of these measurements are given in table 2. The addition of 0.05% of L-histidine, which completely prevented the growth depression caused by the amino acid mixture lacking histidine, greatly stimulated food intake. The histidine intake of the control group was 0.99 gm during the 2-week period as compared with 0.29 gm for the group of rats fed the imbalanced diet. However, the histidine intake of the group of rats ingesting the diet containing the complete amino acid mixture (1.21 gm) was greater than that of the control group even though they gained no more weight. The results of the experiments in which the other amino acids were studied were not as clear-cut but similar trends were observed when leucine, isoleucine and valine were the limiting amino acids.

The results presented in table 3 show the influence of the dietary level of fibrin on the severity of imbalances induced in two different ways. The addition of methionine and phenylalanine caused a marked growth depression only when the diet contained 6% of fibrin. These amino acids failed to cause imbalances even when they were added in greater amounts to diets containing higher levels of fibrin. With only 4% of fibrin in the diet the growth retardation was much less than that obtained when the diet contained 6% of fibrin. In contrast, an imbalance was induced by the mixture of amino acids lacking histidine with each level of fibrin tested. The growth depressions were more pronounced when the diets contained lower levels of fibrin. With 4% of fibrin in the diet, growth was completely prevented, but when the fibrin level was increased to 12%, there was only a slight growth depression.

TABLE 2

Influence of amino acid imbalance on intake of limiting amino acid[1]

	Diet	Gain in weight	Diet consumed	Intake of limiting amino acid
		gm/2 weeks	*gm/2 weeks*	*gm/week*
				Histidine
1	6% Fibrin	33.2 ± 1.8[2]	620	0.99
2	6% Fibrin + amino acid mixture lacking histidine	2.2 ± 1.7	186	0.29
3	Diet 2 + 0.05% L-histidine	32.8 ± 1.9	575	1.21
				Leucine
4	6% Fibrin	28.2 ± 1.1	618	2.77
5	6% Fibrin + amino acid mixture lacking leucine	6.8 ± 1.4	428	1.71
6	Diet 5 + 0.1% L-leucine	24.2 ± 3.5	557	2.78
7	Diet 5 + 0.2% L-leucine	47.2 ± 4.1	586	3.51
				Isoleucine
8	6% Fibrin	30.2 ± 1.0	614	1.72
9	6% Fibrin + amino acid mixture lacking isoleucine	5 ± 2.3	287	0.80
10	Diet 9 + 0.2% DL-isoleucine	38.2 ± 2.1	630	2.39
				Valine
11	6% Fibrin	31.2 ± 1.4	629	1.95
12	6% Fibrin + amino acid mixture lacking valine	8.2 ± 1.4	356	1.10
13	Diet 12 + 0.2% DL-valine	36.8 ± 2.6	542	2.22

[1] See footnote to figure 1 for the composition of complete amino acid mixture.

[2] Standard error of the mean for 5 rats.

TABLE 3

Influence of level of fibrin on imbalance induced by (1) methionine and phenylalanine and (2) by amino acid mixture lacking in histidine

Amino acids added to diet	Weight gain per two weeks				
	4% fibrin	6% fibrin	8% fibrin	10% fibrin	12% fibrin
	gm	*gm*	*gm*	*gm*	*gm*
(A)					
None	22.4 ± 1.6[1]	35.4 ± 2.0	45.0 ± 2.4	60.8 ± 1.4	
0.2% DL-methionine + 0.3% DL-phenylalanine	20.6 ± 2.5	22.4 ± 1.7	49.2 ± 2.4	63.8 ± 2.9	
0.6% DL-methionine + 0.9% DL-phenylalanine	—	—	51.4 ± 9.0	64.6 ± 1.2	
(B)					
None	16.2 ± 1.6	32.6 ± 3.5	56.8 ± 3.1	71.8 ± 3.0	77.6 ± 2.1
Amino acid mixture lacking histidine[2]	−1.4 ± 2.0	13.0 ± 1.9	30.4 ± 2.1	47.2 ± 1.9	72.6 ± 1.6

[1] Standard error of the mean for 5 rats.
[2] See footnote to figure 1 for complete amino acid mixture.

DISCUSSION

Apparently a delicate balance exists among the limiting amino acids, methionine, phenylalanine, leucine, isoleucine, valine and histidine in diets containing 6% of fibrin. A rat, which can metabolize the excess of amino acids in a diet containing 60% of well-balanced protein without any obvious ill effects, suffers a severe growth retardation when it consumes an excess of as little as 0.25 to 0.5% of a mixture of methionine and phenylalanine in a diet containing 6% of fibrin. Furthermore, the growth retardation is not much greater when the level of the mixture of these two amino acids is raised to one to 1.5% of the diet. Preliminary results from further experiments suggest that the effect is quite specific for methionine and phenylalanine. Although the immediate reason for the growth retardation is not known, the addition of these two amino acids apparently causes a depression in the utilization of the next most limiting amino acids, leucine, isoleucine, valine and histidine, because an increase in the dietary levels of these corrects the imbalance.

A more pronounced growth retardation occurs when a larger quantity of an amino acid mixture, lacking only one of the limiting amino acids, is included in the diet. As shown above, the inclusion of an amino acid mixture lacking histidine, leucine, isoleucine or valine causes a marked drop in food intake and, therefore, in the intake of the limiting amino acid. When the level of the limiting amino acid in the diet is increased, food intake rises as well as the intake of the limiting amino acid. Such results suggest that under these conditions, too, poor utilization of the limiting amino acid is responsible for the depression in food intake and consequently, for the growth retardation caused by an amino acid imbalance.

The observations on the relationship between the dietary level of fibrin and the severity of the effects of the imbalances suggest that qualitative differences exist between imbalances induced by a large quantity of an amino acid mixture lacking in a single amino acid and imbalances induced by small supplements of one or two amino acids. The relationship between the dietary level of fibrin and the severity of the imbalance caused by an amino acid mixture lacking in histidine resembles that observed in earlier experiments with diets containing casein. In these, the magnitude of the growth depression, in grams, was greatest when the diet supported about one-half the maximal rate of gain; but on a percentage basis, the growth depression decreased as the protein level was increased (Harper, '59). The minimum amount of L-histidine required to overcome the growth depression caused by adding the amino acid mixture lacking histidine to a diet containing 6% of fibrin, was only 0.05%. Nevertheless, although 0.05% of L-histidine was provided by rais-

ing the dietary level of fibrin from 6 to 8%, the amino acid mixture lacking histidine caused a retardation of growth even when 8% of fibrin was included in the diet. In contrast, although the addition of methionine and phenylalanine to a diet containing 6% of fibrin caused a retardation of growth, no such effect was observed when the dietary level of fibrin was increased to 8%. Apparently, if the diet contains a minimal amount of leucine, isoleucine, valine and histidine, the addition of methionine and phenylalanine causes no adverse effect. A similar type of imbalance between threonine and tryptophan in diets containing 9% of casein but lacking niacin is well known (Hankes et al., '49) and other examples of such delicate balances between the first and second limiting amino acids in diets low in protein have been observed (Henderson et al., '53; Deshpande et al., '55). There is no evidence to indicate that the immediate causes of imbalances produced in these two ways are different but the possibility that they are is worthy of further investigation.

SUMMARY

Amino acid imbalances have been induced in diets containing low levels of fibrin by the addition of (1) relatively small amounts of methionine and phenylalanine and (2) amino acid mixtures lacking in a single amino acid.

The imbalance induced by the former method can be observed when as little as 0.25 to 0.5% of a mixture of methionine and phenylalanine is added to a diet containing 6% of fibrin. The growth depression can be corrected by supplementing this diet with relatively small amounts of the limiting amino acids, leucine, isoleucine, valine and histidine.

The effect of an imbalance in increasing the need for the limiting amino acid has been examined by using amino acid mixtures lacking in leucine, isoleucine, valine or histidine and measuring the growth responses to graded levels of each of these in turn. Measurements of food intake in these experiments indicated that an imbalance reduces the efficiency of utilization of the limiting amino acid.

The depression in growth caused by the addition of methionine and phenylalanine occurs only when the diet contains 6% of fibrin and involves a delicate balance with the next limiting amino acids leucine, isoleucine, valine and histidine. In contrast, the growth depression caused by an amino acid mixture lacking histidine can be demonstrated over a range of fibrin levels.

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Some Observations on Vitamin E Deficiency in the Guinea Pig[1]

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Investigations of metabolic changes associated with the muscular dystrophy of vitamin E deficiency in the rat and guinea pig are tedious because of the time required for the development of this defect and the difficulty in detecting the initial signs of muscular degeneration. The observation that hepatic necrosis, which occurs in rats fed on diets deficient in vitamin E (Hock and Fink, '43), develops rapidly when the vitamin E-deficient diets contain American torula yeast (Schwarz, '51a) suggested that the inclusion of this yeast might speed the development of vitamin E deficiency in the guinea pig. Also the knowledge that in both the rat (György and Rose, '49) and the chick (Muytjens, '56; Gitler, Sunde and Baumann, '58) vitamin E deficiency leads to a susceptibility of erythrocytes to lysis by dialuric acid suggested that this defect might serve as an indicator of vitamin E depletion and hence the initiation of the muscular degeneration of vitamin E deficiency in the guinea pig.

The results reported in this paper show that American torula yeast fed at a level of 20% in the diet retards rather than enhances the development of vitamin E deficiency in the guinea pig. However, evidence from a study of the relative rates of development of the various signs of vitamin E deficiency indicates that the time required for the development of susceptibility to hemolysis by dialuric acid in individual guinea pigs can be used as an indicator of the rate of development of the other signs of vitamin E deficiency.

Some observations on the adenosinetriphosphatase (ATPase) activity of muscle from vitamin E-deficient guinea pigs are also included. Although the amount of adenosinetriphosphate (ATP) in skeletal muscle from vitamin E-deficient rabbits was found to be low (Feuer and Frigyes, '52), reports of the effect of vitamin E deficiency on ATPase activity in muscle have been conflicting. Carey and Dziewiatkowski ('49) and Jacobi et al. ('50) reported the activity to be normal whereas Hummel ('48) and Feuer and Frigyes ('52) found the activity of this enzyme to be low. No evidence was obtained in the experiments described in this paper of a statistically significant depression in muscle ATPase as a result of vitamin E deficiency.

METHODS

One-week old guinea pigs were used in the experiments, and fed a vitamin E-deficient diet based on that of Heinecke, Harper and Elvehjem ('55). The diet contained in per cent: sucrose, 37.2; alcohol-extracted casein, 30; powdered cellulose,[2] 15; salts (Harper, '59) 6; antioxidant free lard,[3] 5; cod liver oil,[4] 3; potassium acetate, 2.5; magnesium oxide, 0.5; choline chloride, 0.35; ascorbic acid, 0.20; and water soluble vitamin mixture, 0.25. The vitamin mixture[5] contained (parts by weight): inositol, 200; niacin, 20; *p*-aminobenzoic acid, 10; calcium pantothenate, 8; ribo-

Received for publication July 23, 1959.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

[2] Solka Floc BW200, Brown Company, Chicago.

[3] Kindly supplied by Dr. B. P. Haasl of the Department of Animal Husbandry.

[4] Contained 1800 USP units of vitamin A and 175 USP units of vitamin D/gm. Obtained from Mead Johnson Co., Evansville, Ind.

[5] We are indebted to Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey for some of the crystalline vitamins.

flavin, 3; thiamine, 2; pyridoxine, 2; folic acid, 1; menadione, 0.2; biotin, 0.1; and cyanocobalamin, 0.004. When torula yeast was included, the diet contained in per cent: torula yeast,[6] 20; sucrose, 27.2; alcohol-extracted casein, 20; and all other components in the amounts listed above. Normal control animals were supplemented with 20 mg of alpha tocopherol in corn oil three times per week.

Urine was collected by placing animals in metabolism cages for 6-hour periods from 12 noon to 6 P.M. with water, but without food (since otherwise food spillage contaminated the urine samples). Urine was frozen and stored at −12°C for the analyses. Creatine and creatinine were analyzed by a modification of the Folin method (Folin, '04) outlined by Braun ('54).

Blood was obtained by heart puncture from animals anesthetized with ether. Hemolysis by dialuric acid prepared according to Biltz and Damm ('13), was determined by the method of Rose and György ('52).

Adenosinetriphosphatase activity was determined on homogenates of the gastrocnemius muscle by a method based on that of DuBois and Potter ('43) with the following modifications: a 1% (w/v) muscle homogenate in 0.25 M of sucrose was used; 0.1 M of trishydroxymethylaminomethane[7] buffer, pH 7.4, was substituted for diethyl barbiturate; and the incubation mixture contained 3.9 μm of ATP. Activation with magnesium was measured using 6.5 μm of $MgSO_4$ per tube. Inorganic phosphorus was determined by the method of Fiske and SubbaRow ('25), and non-collagen nitrogen by the method of Lillienthal et al. ('50), except that a microKjeldal procedure was used for nitrogen determination.

RESULTS

Effect of torula yeast on muscular dystrophy. Growth curves for guinea pigs fed the basal diet with and without vitamin E are shown in figure 1. During the first 8 weeks of the experiment the growth rates of the two groups were not significantly different, but at the end of 11 weeks the growth rate of the group fed the vitamin E-deficient diet had declined. At this time

[6] Lake States Yeast Corp., Rhinelander, Wisconsin.

[7] Trishydroxymethylaminomethane and ATP were obtained from Sigma Biochemical Corp., St. Louis.

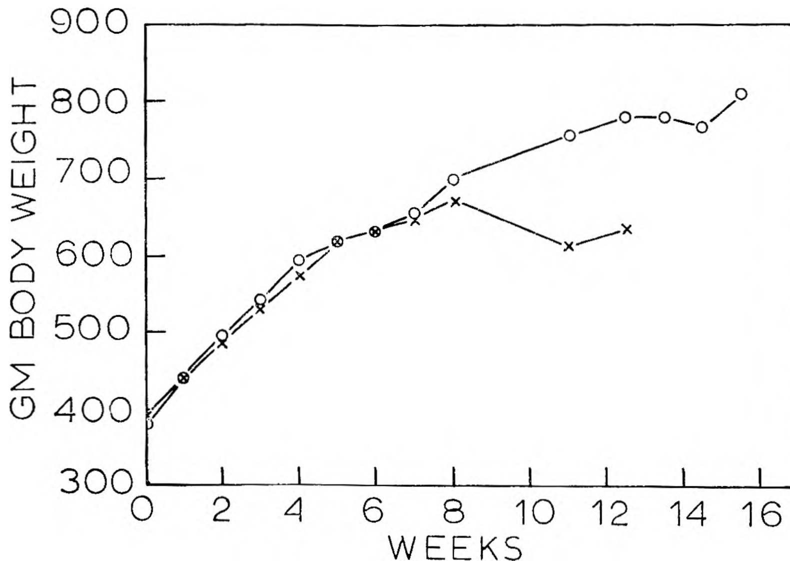

Fig. 1 Effect of vitamin E deficiency on rate of gain of guinea pigs fed purified diets. Two groups of 8 animals were fed the basal diet. Points marked by O indicate animals fed diets supplemented with 60 mg of α-tocopherol per week, and points marked by X indicate deficient animals.

the animals in this group had developed creatinuria and showed a delay in righting themselves when placed on their backs.

The effect on the growth rates of including torula yeast in these diets is shown in figure 2. No significant difference was observed between the growth rate of the group not receiving vitamin E and that of the supplemented control group when the diets contained 20% of torula yeast. During the first week of the experiment the groups fed the diets containing torula yeast gained less rapidly than the groups fed the basal diet. From the second week to the end of the 15th week the rates of growth of animals fed the basal diet with vitamin E and those fed the diets containing torula yeast, with or without vitamin E, were not significantly different. During the 16th week, however, the growth rate of animals receiving torula yeast was lower than that of those fed the basal, control diet.

Creatinuria occurred after 11 weeks in animals fed the vitamin E-deficient basal diet as shown in table 1. Three of the 8 animals in this group had died before the end of 13 weeks. Animals fed the diet containing torula yeast but no vitamin E showed no increase in urinary creatine: creatinine ratios until the 15th week of experiment. None of the animals in this group succumbed to muscular dystrophy before the experiment was terminated after 16 weeks.

The animals fed the basal diet without vitamin E showed a delay in righting themselves when placed on their backs, which occurred shortly after creatinuria appeared. In several instances, animals fed the torula yeast diet showed a slight delay in righting themselves when placed on their backs; however, this occurred with both vitamin E-deficient and control animals.

Although the addition of torula yeast to the vitamin E-deficient diet delayed the appearance of signs of vitamin E deficiency, feeding the diet to two vitamin E-deficient animals did not alleviate the deficiency signs.

Dialuric acid hemolysis and muscular dystrophy. Preliminary experiments showed that blood from vitamin E-deficient guinea pigs was hemolyzed by dialuric acid and that the hemolysis was prevented by administration of vitamin E to the deficient animals. Therefore, the time of onset of

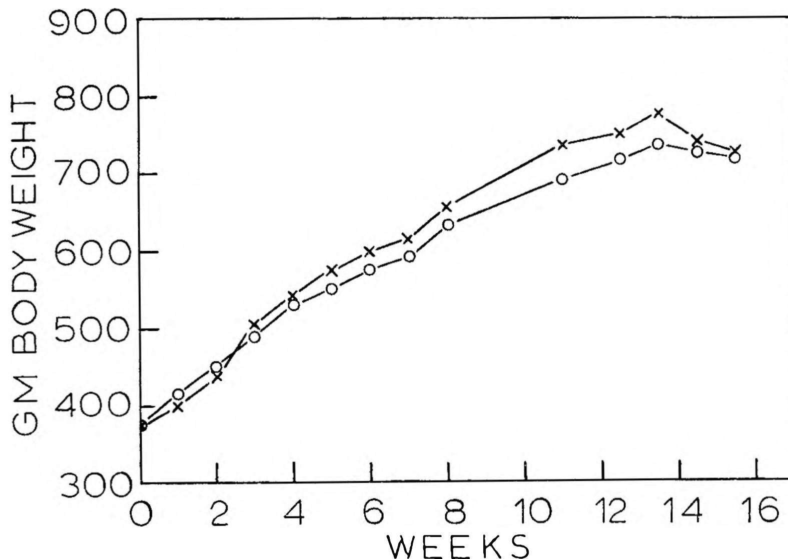

Fig. 2 Effect of vitamin E deficiency on rate of gain of guinea pigs fed diets containing torula yeast. Two groups of 8 animals were fed the diet containing 20% of torula yeast. Points marked by O indicate animals supplemented with 60 mg α-tocopherol per week, and points marked by X indicate deficient animals.

dialuric acid hemolysis was determined and compared with that of the onset of signs of muscular dystrophy. Nine animals were fed the basal diet without vitamin E until hemolysis was demonstrated, and at this time they were supplemented with 20 mg of α-tocopherol. They were then fed the vitamin E-deficient basal diet until they succumbed to muscular dystrophy.

Because the values for successive determinations of hemolysis on blood from animals which apparently were depleted of vitamin E varied between 75 and 100%, values of 75% hemolysis or greater were taken to indicate depletion of vitamin E and are referred to as extensive hemolysis.

Table 2 shows the individual variation in the appearance of extensive hemolysis. With the exception of animal 7, the order in which individual animals developed a susceptibility to hemolysis was the same during the initial depletion period and after depleted animals were supplemented with α-tocopherol. Figure 3 illustrates the onset and development of hemolysis as the average of 9 animals fed the basal diet with no vitamin E.

The time that elapsed between the first detectable hemolysis and extensive hemolysis varied from three days in animals 2, 3 and 8, to 9 days in animal 6.

A peculiar pattern in the development of hemolysis exhibited by animals 1 and 9

TABLE 1

Effect of dietary torula yeast on urinary creatine: creatinine ratios in vitamin E deficiency

Experimental group[1]	Days of experiment					
	77		96		103	
	No. animals	Creatine: creatinine	No. animals	Creatine: creatinine	No. animals	Creatine: creatinine
1	2	0.10	5	0.10 ± 0.03		
2	8	0.70 ± 0.25[2]				
3	7	0.05 ± 0.02	8	0.20 ± 0.04	2	0.15
4	6	0.10 ± 0.02	6	0.20 ± 0.09	6	0.35 ± 0.07

[1] Group 1 was fed the basal diet + 60 mg α-tocopherol per week, group 2 was fed the basal diet without vitamin E, group 3 was fed the diet containing 20% of torula yeast + 60 mg of α-tocopherol/week and group 4 was fed the diet containing 20% of torula yeast with no vitamin E.

[2] Standard error of the mean.

TABLE 2

The onset of symptoms of vitamin E deficiency in the guinea pig[1]

Animal	Day of onset of extensive hemolysis		Day of onset of creatinuria	Day of onset of physical weakness[3]	Day of onset of disability	Day of death
	Initial	After supplementation[2]				
3	25	10	26	—	—	42
7	25	17	35	<42	42	46
9	25	14	35	42	46	49
8	25	14	39	43	47	49
2	25	14	39	47	55	57
1	25	23	63	61	66	67
5	28	23	59	42	66	68
4	31	23	51	61	—	—
6	34	23	63	66	—	—

[1] The onset of symptoms of vitamin E deficiency is recorded as the number of days after supplementation except for initial onset of hemolysis.

[2] Diets were supplemented with 20 mg of α-tocopherol on the 45th day.

[3] Physical weakness denotes a delay in righting itself when animal was placed on its back and disability denotes the inability to do so.

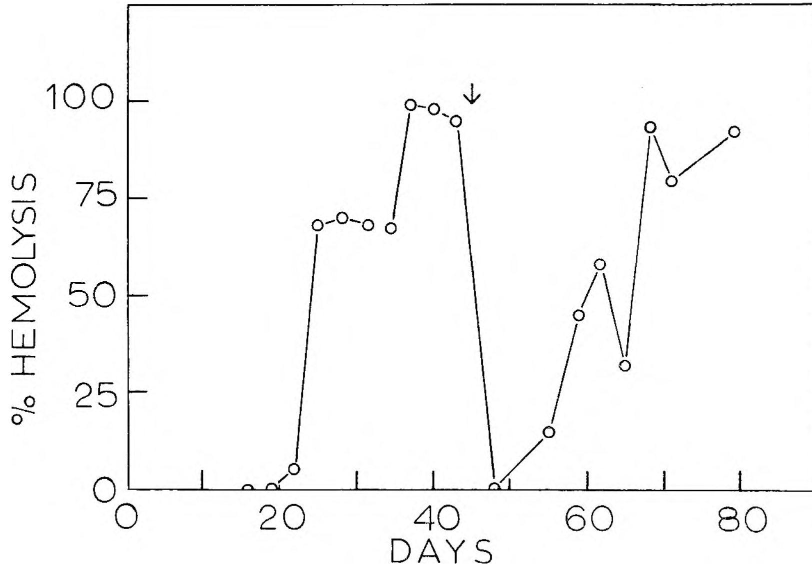

Fig. 3 Effect of vitamin E deficiency on susceptibility of guinea pig erythrocytes to lysis by dialuric acid. Nine animals were fed the vitamin E-deficient basal diet. Supplementation with 20 mg of α-tocopherol per animal is indicated by the arrow. Six control animals fed diets supplemented with 60 mg of α-tocopherol per animal per week showed no hemolysis at any time during the experiment.

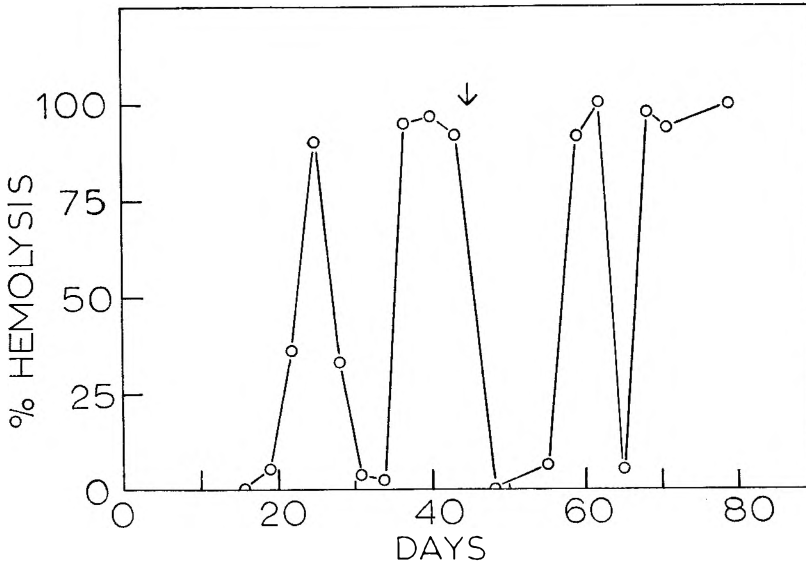

Fig. 4 Extreme individual variation in susceptibility of erythrocytes to lysis by dialuric acid. Animal 9 showed this pattern of development of hemolysis which was characteristic of several other animals. Supplementation with 20 mg of α-tocopherol is indicated by the arrow.

is shown in figure 4. After hemolysis had risen to 85 and 90%, respectively, on the 28th day, a decrease to 5% hemolysis was noted and values did not rise again until the 37th day. After vitamin E-depleted animals were supplemented with α-tocopherol, a similar pattern was exhibited by animals 6 and 9 and to a lesser extent by animals 2, 5 and 7. These low values for hemolysis account for the apparent plateau in figure 3 for the average time of development of hemolysis from the 25th to the 34th day.

A comparison between the onset and development of dialuric acid hemolysis and the onset of creatinuria and physical signs of muscular dystrophy (delay of animal in righting itself when placed on its back and the inability to do so) can be made from the values in table 2. The order in which animals showed susceptibility to extensive hemolysis was similar to that in which creatinuria, physical signs of muscular dystrophy and death occurred. The order in which animals developed a susceptibility to extensive hemolysis correlated very well with that of the onset of disability and the time of death. The only exception to this correlation appeared after depleted animals were supplemented with α-tocopherol when animal 7 required a slightly longer period of time for the appearance of extensive hemolysis than animals 2, 8 and 9; whereas during the initial depletion period, animal 7 showed extensive hemolysis on the 25th day of the experiment as did animals 2, 8 and 9.

Animals 4 and 6 were the only vitamin E-defiicent animals surviving until the 68th day, when the experiment was terminated. At this time, both animals had developed creatinuria and showed a delay in righting themselves when placed on their backs. Animal 4 appeared more severely dystrophic than animal 6 and had shown creatinuria for a period of 17 days compared with 5 days for animal 6.

Creatinuria was considered to be present when creatine:creatinine ratios exceeded 0.1 since the ratio did not exceed 0.1 in any of the control animals at any time.

Susceptibility to extensive hemolysis did not correlate as well with the onset of creatinuria as with the onset of disability and time of death. The order in which susceptibility to extensive hemolysis developed in animals 1, 5 and 4 was the reverse of that in which these three animals developed creatinuria. Observations of their behavior and those of the behavior of animal 7 (pointed out above) were the only exceptions to a direct relationship between the time of development of a susceptibility to hemolysis and the onset of creatinuria.

Animals 5 and 7 were the only exceptions to a direct relationship between the development of susceptibility to hemolysis and the onset of physical weakness. Physical weakness appeared relatively early in animal 5, even before consistently high creatine:creatinine ratios were observed.

Several vitamin E-deficient animals showed a delay in righting themselves after having been placed on their backs several times in succession even before creatinuria appeared.

Table 2 shows that smaller individual variations were observed in the time at which susceptibility to extensive hemolysis could be demonstrated than in the time of onset of creatinuria or physical symptoms of muscular dystrophy. The period of time from the detection of susceptibility to hemolysis in animal 3 until all animals showed a similar susceptibility to hemolysis was 14 days during the initial depletion period and 13 days after depleted animals had been supplemented with vitamin E. The corresponding period for the onset of creatinuria was 37 days. For the appearance of physical weakness, disability and death of the animals, the corresponding period was at least 26 days. The appearance of physical weakness and disability was not noted in animal 3, and animals 4 and 6 showed no disability at the end of the experiment.

ATPase activity of muscle in vitamin E deficiency. The ATPase activity of muscle homogenates from vitamin E-deficient animals was slightly lower than that of homogenates from normal control animals when the activity was expressed per milligram of fresh tissue as indicated in table 3. This trend was evident when no activator was added and

TABLE 3

Effect of vitamin E deficiency on ATPase activity of muscle[1]

	Inorganic P liberated per mg of fresh tissue		Inorganic P liberated per mg of non-collagenous nitrogen	
	Normal control group	Vitamin E-deficient group	Normal control group	Vitamin E-deficient group
	μm	μm	μm	μm
Activity with Ca	0.26 ± 0.027[2]	0.23 ± 0.029	1.81 ± 0.31	1.63 ± 0.29
Activity with Mg	0.33 ± 0.027	0.26 ± 0.026	1.93 ± 0.30	1.86 ± 0.60
Activity without added activating ions	0.13 ± 0.015	0.11 ± 0.015	0.95 ± 0.18	0.78 ± 0.19
Increase in activity due to Ca	0.14 ± 0.018	0.12 ± 0.018	0.86 ± 0.16	0.85 ± 0.09
Increase in activity due to Mg	0.17 ± 0.016	0.15 ± 0.013	1.00 ± 0.15	1.08 ± 0.13

[1] ATPase activity of muscle homogenates from guinea pigs in advanced stages of muscular dystrophy was measured with Ca activation, Mg activation and with no added activating ion.

[2] Standard error of the mean.

also when either calcium or magnesium ions were added to the assay tubes.

Very little trend toward lower ATPase activity in homogenates from vitamin E-deficient animals was shown when activity was expressed per milligram of non-collagenous nitrogen. It should be noted however that the differences expressed on either basis are not statistically significant.

The difference between ATPase activity measured in the presence of activating ions, and activity in the absence of added activators, was not appreciably affected by vitamin E deficiency.

DISCUSSION

Effect of torula yeast on muscular dystrophy. The inclusion of torula yeast in the basal diet delayed the onset of muscular dystrophy in the guinea pig but did not prevent elevation of the urinary creatine: creatinine ratios. It has recently been demonstrated that the necrogenic properties of torula yeast are the result of the low content of selenium[8] (Gitler et al., '57; Schwarz, '51b). In preliminary experiments sodium selenite was found to be without effect on muscular dystrophy in the guinea pig, and Draper ('57) and Hove, Fry and Schwarz ('58) have shown that muscular dystrophy of vitamin E deficiency in the rabbit is not affected by sodium selenite or selenocystine.

Torula yeast contains coenzyme Q, a substance believed to function in electron transport,[9] but subcutaneous injection of coenzyme Q[10] isolated from torula yeast (1 to 4 mg/animal/day) did not alleviate muscular dystrophy in vitmain E-deficient guinea pigs.

Since torula yeast is reported to contain one or more substances having antioxidant properties and protecting against hemolysis by dialuric acid (Cowlishaw and Prange, '57; Forbes et al., '58), it is possible that such substances may protect vitamin E against oxidative destruction and in this way delay the onset of muscular dystrophy.

The depression of the growth rate of animals fed torula yeast which occurred during the first week was probably the result of a lack of acceptance of the new diet. A similar growth depression was consistently observed in guinea pigs transferred from a natural diet to a synthetic diet.

[8] Schwarz, K., C. M. Foltz 1957 Selenium as an integral part of Factor 3 against dietary necrotic liver degeneration. J. Am. Chem. Soc., *79:* 3292 (communication to editor).

[9] Lester, R. L., F. L. Crane and Y. Hatefi 1958 Coenzyme Q: A new group of quinones. J. Am. Chem. Soc., *80:* 4751 (communication to editor).

[10] Coenzyme Q from torula yeast was kindly supplied by Dr. R. L. Lester of the Enzyme Institute, Madison, Wis.

It is not clear whether the loss of weight which occurred after 14 weeks in animals fed torula yeast was an effect of torula yeast or some other cause, since vitamin E-supplemented animals fed the basal diet also showed a loss of weight during this period.

Comparison of dialuric acid hemolysis and muscular dystrophy. A direct relationship appears to exist between the time required for susceptibility to extensive hemolysis to develop and the time of onset of other symptoms of vitamin E deficiency.

Although the appearance of extensive hemolysis did not correlate as well with the onset of physical weakness as with the onset of disability and the time of death, it should be pointed out that the detection of physical weakness is less reliable than the detection of other signs of vitamin E deficiency. The exact point at which the delay of an animal in righting itself from its back first occurs is difficult to determine. Also, this test is undoubtedly influenced by factors other than vitamin E deficiency.

Values of hemolysis varied considerably in any individual animal after values of 75% were observed. Friedman et al. ('58) have reported that the determination of hemolysis by dialuric acid is sensitive to factors such as dilution of erythrocytes. Some of the variation in our results may be owing to such factors.

Since hemolysis occurs only in animals fed a vitamin E-deficient diet for at least three weeks and is prevented when vitamin E is administered to these animals, the onset of hemolysis appears to indicate the depletion of vitamin E and the starting point for the series of changes leading to muscular dystrophy in vitamin E-deficient animals. No explanation can be provided for the subsequent decrease in hemolysis to nearly zero following the initial rise to values of 75%, or greater, which occurred in some animals.

Adenosinetriphosphatase activity of muscle in vitamin E deficiency. The process of muscular degeneration in vitamin E deficiency was shown by Azzone and Aloisi ('58) to decrease the amount of myosin and actomyosin extractable from rabbit muscle. Since the magnesium-activated ATPase of muscle is strongly inhibited by calcium (Kielley and Meyerhof, '48), the ATPase activity of muscle homogenates measured in the presence of calcium should be a measure of the ATPase activity of myosin or actomyosin. ATPase activity measured in the presence of magnesium follows the same trend but is higher than in the presence of calcium, possibly as a result of the magnesium-activated ATPase. The trend toward lower ATPase activity of muscle in vitamin E deficiency is very slight and of doubtful significance when activity is expressed per milligram of noncollagenous nitrogen while there is a slightly greater trend toward lower values in vitamin E deficiency when activity is expressed per milligram of fresh tissue. Any decrease in ATPase activity of muscle in vitamin E deficiency appears to be a result of the loss of myosin and actomyosin accompanying the dystrophic process as concluded by Feuer and Frigyes ('52). The previous work, indicating no difference between ATPase activity of muscle from normal and vitamin E-deficient rabbits, was done with animals in the early stages of muscular dystrophy when the degeneration of muscle and consequent loss of myosin and actomyosin is slight; whereas the guinea pigs used in our work exhibited signs of advanced muscular dystrophy. Carey and Dziewiatkowski ('49) reported that one animal in the later stages of muscular dystrophy showed a very low ATPase activity of muscle compared with less severely dystrophic animals; and the data of Jacobi et al. ('50) show a trend toward lower ATPase activity in vitamin E deficiency similar to that reported here. Therefore it seems that any decrease in ATPase activity in vitamin E deficiency as measured in muscle homogenates is slight and occurs only in the later stages of muscular dystrophy.

SUMMARY

The inclusion of torula yeast in a vitamin E-deficient diet definitely delays the onset of muscular dystrophy in the guinea pig but will not prevent the disease when fed at a level of 20% in the experimental diet. No effect of coenzyme Q or sodium selenite on muscular dystrophy was shown.

Erythrocytes from vitamin E-deficient guinea pigs become susceptible to lysis by

dialuric acid two to 6 weeks before signs of muscular dystrophy appear and the order in which extensive hemolysis appears in individual animals correlates well with the order in which they develop muscular dystrophy. Extensive hemolysis appears to be an acceptable criterion of depletion of vitamin E for use in studying metabolic defects leading to muscular dystrophy.

The ATPase activity of muscle homogenates shows a slight trend toward lower values in vitamin E deficiency, which appears to be the result of a lowered content of myosin and actomyosin in dystrophic muscle.

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Effects of Marginal and Optimal Intakes of B Vitamins on Protein Utilization by the Growing Rat from Varied Dietaries[1]

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The adverse effect of uneven distribution of the protein and carbohydrate moieties of the diet on nitrogen balance has been well demonstrated in humans (Cuthbertson and Munro, '39; Munro and Wikramanayake, '54), dogs (Larson and Chaikoff, '37; Munro and Wikramanayake, '54), and rats (Cuthbertson, McCutcheon and Munro, '40; Munro, '49; Lathe and Peters, '49; Geiger, Bancroft and Hagety, '50; Munro and Wikramanayake, '54). It has also been shown that with casein (Sure and Romans, '48) and fibrin (Sure, '50) diets, increasing concentrations of B vitamins increase growth and protein utilization. Impaired reproductive function in rats on low-protein diets is known to be improved by raising the level of dietary B vitamins (Nelson and Evans, '53). Equitable distribution of vitamin supplements is also apparently essential for effective feed utilization in poultry (Waibel, Cravens, Bird and Baumann, '54).

It was therefore of interest to ascertain if, in split feeding of the protein and non-protein components of diet, variations in levels of B vitamins have a functional significance in the utilization of proteins in rats. Such considerations will have implications in practical human nutrition where widespread extremes may often exist in the composition and distribution of individual meals (Platt and Miller, '58).

EXPERIMENTAL AND RESULTS

Split feeding of protein and other caloric components of a diet

Adult male Wistar rats 200 to 210 gm in weight were used. The composition of the experimental diets is presented in table 1.

The animals were fed a protein-free "depletion" diet (diet 1), with fasting on alternate days to accelerate mobilization of body protein. After 12 days, when the animals weighed approximately 170 gm, they were divided into 4 comparable groups, receiving the composite (diet 2), or split (diets 3 and 4) rations supplemented with B vitamins at one of two levels, a low minimal and a high optimal. The vitamin and mineral additions were more or less proportionally distributed in the protein and non-protein caloric moieties of the diet in the split-fed groups.

The animals had access to the diets in excess quantities and in scatter-proof cups twice a day at 9.30 A.M. and at 4.30 P.M. The order of feeding the two portions of the split-fed diet was reversed on successive days. Feed-cups were removed after one-half hour and the residues weighed.

Nitrogen retention studies were made during 4, 4-day periods. Animals were placed in individual, round metabolism cages. Urine and feces were collected before the morning feeding. Urine was stored with the addition of a few drops of sulphuric acid and toluene. Feces were dried at 80°C for 24 hours and weighed. The nitrogen content of the excreta pooled for each 4-day period was determined by the Kjeldahl method. In table 2 are presented the data on nitrogen retention and efficiency.

In order to obviate the effects of differences in food intake, the experiment was repeated with isocaloric feeding. The protein-depleted animals were fed 10 gm of

Received for publication May 25, 1959.

[1] This investigation was supported by a research grant from the Williams-Waterman Fund, Research Corporation, New York.

the diets daily. The split-fed groups received this quantity in two installments, 2.3 gm of the protein moiety (diet 3) in one meal and 7.7 gm of the caloric moiety (diet 4) in the following meal; both portions were proportionately represented with respect to the vitamin-mineral supplements (table 1). The results are included in table 2.

In animals fed ad libitum and given the minimal as contrasted with optimal amounts of B vitamins (table 2), split feeding markedly influenced the rate of protein repletion, evidenced by the well-defined differences in the data for nitrogen retention, weight gain and nitrogen efficiency, the increases being 3.5, 4.7 and 1.5 times the "minimal" values, respectively. This was also true under the conditions of restricted (isocaloric) feeding, but in this case the increases were more nearly the same, being 2.1, 2.8 and 2.7 times the "minimal" values for nitrogen retained, weight gained and nitrogen efficiency, respectively.

These studies were extended to a rice-legume diet based on a typical Indian vegetarian meal (table 1). The composite

TABLE 1

Composition of the diets used

Component	Diet no.[1]									
	Depletion	Experimental								
	1	2	3	4	5	6	7	8	9	10
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Devitaminized casein	—	20	20	—	—	—	—	10	7.7	—
Corn starch	65	65	—	65	—	—	—	—	19.3	—
Cellulose[2]	5	5	1.2	3.8	—	—	—	—	—	—
Dextrin	20	—	—	—	—	—	—	—	—	—
Vitaminized sucrose‡	1	1	0.2	0.8	—	—	—	1	1	1
Vitaminized arachis oil†	5	5	1.2	3.8	5	2.55	2.45	5	5	5
Salt mixture[3]	4	4	0.9	3.1	—	—	—	4	4	4
Rice	—	—	—	—	37.5	—	37.5	45	35	90
Legume[4]	—	—	—	—	30	30	—	35	28	—
Fresh milk	—	—	—	—	10	10	—	—	—	—
Non-leafy vegetables[5]	—	—	—	—	10	5.1	4.9	—	—	—
Leafy vegetables[6]	—	—	—	—	5	2.55	2.45	—	—	—
Spice mixture[7]	—	—	—	—	2.5	1.3	1.2	—	—	—

‡ B vitamins per gm sucrose	Optimal	Minimal
	mg	*mg*
Thiamine·HCl	0.3	0.015
Riboflavin	0.4	0.015
Pyridoxine·HCl	0.3	0.015
Ca pantothenate	1.0	0.1
Niacin	2.0	0.1
Biotin	0.05	nil
Folic acid	0.1	0.003
Vitamin B_{12}	0.015	nil
Inositol	20.0	5.0
Choline Cl	20.0	5.0
Menadione	0.5	0.5

† Fat-soluble vitamins per 5.0 gm arachis oil	
	mg
Vitamin A acetate	0.31
Vitamin D (calciferol)	0.0045
Alpha tocopherol	5.0

[1] Additions of B vitamins to diets 2, 3, 4 and 8, 9, 10 were either at optimal or at minimal levels. Diets 5, 6, 7 were with or without supplements of B vitamins at the optimal level. B vitamins in the "depletion diet" were at optimal levels.

[2] Acid-washed powdered filter paper.

[3] U.S.P. XIV salt mixture.

[4] *Cajanus indicus.*

[5] The non-leafy vegetables used were edible portions of eggplant and okra in equal proportion.

[6] Leafy vegetables included amaranth and spinach in equal proportion.

[7] The spice mixture consisted of (parts): sodium chloride, 1; tamarind, 0.7; pepper, 0.1; chillies, 0.1; tumeric, 0.1; and cinnamon, 0.1; with additions of calcium (as calcium lactate) and iron (as ferrous ammonium sulphate) at levels of 50 mg and 2 mg respectively per 100 gm of the spice mixture.

TABLE 2

Effect of variations in dietary levels of B vitamins on nitrogen balance and nitrogen efficiency in protein-depleted rats refed the protein and caloric moieties of the diet separately at intervals (split-ration) or concurrently (composite ration)

Ration	Level of B vitamins[1]	Nitrogen balance[2]			Weight gain[3]	Nitrogen efficiency[4]
		Nitrogen intake	Caloric intake	Nitrogen retained		
		mg	*Cal.*	*mg*	*gm*	
Ad libitum feeding						
Split	Minimal (6)	355 ± 21[5]	41 ± 2	58 ± 13	27 ± 3	4.8 ± 0.9
Split	Optimal (5)	1088 ± 75	68 ± 3	204 ± 37	128 ± 7	7.3 ± 0.7
Composite	Minimal (6)	342 ± 21	44 ± 3	91 ± 14	46 ± 5	8.3 ± 0.7
Composite	Optimal (5)	567 ± 14	71 ± 2	222 ± 13	137 ± 6	15.1 ± 1.1
Restricted (isocaloric) feeding[6]						
Split	Minimal (6)	306 ± 13	38 ± 2	52 ± 8	20 ± 3	4.1 ± 0.3
Split	Optimal (6)	320 ± 4	40 ± nil	108 ± 3	56 ± 6	10.9 ± 0.8
Composite	Minimal (6)	301 ± 9	38 ± 1	92 ± 8	24 ± 3	5.0 ± 0.4
Composite	Optimal (6)	320 ± 4	40 ± nil	121 ± 4	88 ± 8	17.1 ± 0.7

[1] Figures within parentheses indicate the number of animals in each group.

[2] Figures represent mean values per rat per day and are calculated from data obtained for each animal of the group over 4 separate periods of 4 days' duration each.

[3] Mean gains per rat over entire period of 16 days of nitrogen balance study.

[4] Mean grams gain per day per gram of nitrogen consumed.

[5] Standard error of the mean.

[6] The small differences in caloric intake are due to traces of left-over food.

diet (diet 5) provided 320 Cal. per 100 gm and 15.1 % of protein, which was split between the predominantly protein component (legume-milk) (diet 6) and the predominantly carbohydrate component (rice) (diet 7) in the proportion 11.2:3.9. The composite and split rations were fed with or without a supplement of B vitamins at the optimal level, as indicated in table 1. Distribution of the vitamin supplement between the protein and carbohydrate moieties of the split ration was in the proportion of 1:4. During the experimental regeneration period feeding was ad libitum.

The data presented in table 3 again indicated an impairment of nitrogen retention as a result of separating the major protein and carbohydrate moieties of the diet. A similar effect, although less marked, was manifested in the growth rate. Nitrogen retention was improved markedly with vitamin supplementation and the deleterious effect of split feeding was reduced simultaneously. Nitrogen efficiency was, apparently, not influenced by the feeding procedure.

Intermittent feeding of proteins from a rice-legume-casein diet

In the next experiment, the effects of alterations in the level of protein feeding from day to day was studied in relation to dietary levels of B vitamins. The composition of the diets used is shown in table 1. Weanling male rats, 40 gm in weight, were fed alternately on diets with 18% of protein (diet 8) and 6% of protein (diet 10) on successive days. Such short-term protein restriction does not create a specific protein hunger in the rat (Geiger, Rawi and Thomas, '55). A ration providing 14% of protein (diet 11) was offered daily to the control group. The diets were based on rice for the low-protein ration, and on suitable proportions of rice, legume and casein for the two higher protein ones. After thorough mixing, the diets were steam-cooked for 45 minutes, dried at 70°C under vacuum and powdered. Comparable groups of animals received these diets ad libitum, varied with respect to the two levels, minimal and optimal, of the B vitamins. Food consumption and body weight records were recorded throughout the 6-week experimental period.

At the end of 6 weeks blood and sections were removed from the animals under ether anesthesia. Blood obtained from the hepatic portal vein was quickly heparinized and the plasma separated by centrifuging in the cold. Protein-free plasma filtrate was prepared by the procedure of Hier and Bergeim ('45); total and non-protein nitrogen was determined by direct Nesslerization following its initial liberation by Kjeldahl micro-digestion (Umbreit, '46). Nitrogen in liver homogenates and in protein-free preparations (obtained by trichloroacetic acid precipitation of the proteins) of the tissue was similarly determined. Total liver lipide was estimated by the method of Sperry ('54).

TABLE 3

Utilization of a rice-legume diet as influenced by split-feeding of its major protein and carbohydrate moieties and the effects of additional ingestion of a mixture of B vitamins[1]

Ration	B vitamins[2]	Nitrogen balance[3]			Gain in weight[4]	Nitrogen efficiency[5]
		Nitrogen intake	Caloric intake	Nitrogen retained		
		mg	*Cal.*	*mg*	*gm*	
Split	−	211 ± 15	28 ± 3	55 ± 8	33 ± 6	13.0 ± 0.4
Split	+	271 ± 16	41 ± 3	127 ± 9	48 ± 4	14.6 ± 0.3
Composite	−	262 ± 19	35 ± 3	94 ± 10	40 ± 4	12.6 ± 0.5
Composite	+	296 ± 17	40 ± 4	146 ± 9	53 ± 5	14.8 ± 0.5

[1] The data were obtained with 6 animals per group.

[2] The diets were with (+) or without (−) a supplement of B vitamins at optimal levels as indicated in table 1.

[3] Figures are mean values per rat per day with standard errors, calculated from data obtained for each animal of the group over three separate periods of 4 days' duration each.

[4] Mean weight gains per rat with standard errors (12 days).

[5] As defined in table 2.

TABLE 4

Influence of dietary B vitamins on protein efficiency and on certain tissue constituents as affected by uneven feeding of protein from a rice-legume-casein diet[1]

Diet description		Protein efficiency (5 weeks)			Tissue constituents				
					Liver[4]			Plasma	
Protein content[2]	Level of B vitamins	Protein intake	Weight gain	PER[3]	Total nitrogen	Non-protein nitrogen	Total lipids	Total nitrogen	Non-protein nitrogen
%		gm	gm		mg/gm	mg/gm	%	mg/ml	mg/ml
14	Minimal	58 ± 7	70 ± 6	1.21 ± 0.03	90 ± 4	8.8 ± 0.4	28 ± 2	7.3 ± 0.3	0.75 ± 0.04
14	Optimal	61 ± 5	128 ± 8	2.10 ± 0.21	99 ± 4	11.0 ± 1.0	39 ± 2	8.8 ± 0.2	0.49 ± 0.05
18 or 6	Minimal	55 ± 3	45 ± 4	0.81 ± 0.07	88 ± 3	11.1 ± 0.7	34 ± 3	6.9 ± 0.4	1.01 ± 0.04
18 or 6	Optimal	56 ± 5	84 ± 5	1.50 ± 0.12	95 ± 4	11.5 ± 0.5	40 ± 4	7.8 ± 0.2	0.66 ± 0.03

[1] Figures represent in each case, the mean of at least 5 replicates with its standard error.

[2] The animals were maintained either on a uniform diet with 14% protein (diet 9, table 1) or, interchangeably, on diets with 18% protein (diet 8) and 6% protein (diet 10) on successive days.

[3] Protein efficiency ratio, expressed as grams gain per gram of protein consumed.

[4] Values are expressed on the dry weight of the tissue.

The data on protein utilization and on plasma and liver constituents are presented in table 4. With both uniform and varied dietaries an increased intake of the B vitamins caused marked improvements in growth rate and efficiency of protein utilization. Further, these were associated with an increase in protein-nitrogen in liver and in plasma, increase in liver lipides and a decrease in plasma non-protein nitrogen. A varied intake of protein resulted in impaired protein utilization with consequent retardation of growth rate. These effects were not appreciably counteracted by B vitamins offered in optimal amounts. However, the non-uniform intake of protein caused an elevation of non-protein nitrogen in liver and in plasma as also in liver lipides and these effects were considerably suppressed with the higher intake of the vitamins. The reduced content of non-protein nitrogen in liver and in plasma and, hence, a possible reduction of free amino acids in these tissues with higher intake of B vitamins may point to a more efficient utilization of dietary amino acids. A similar specific effect of vitamin B_{12} has been reported in chicks (Charkey, Wilgus, Patton and Gassner, '50).

DISCUSSION

The two levels of B vitamins chosen in the present experiments were close to the optimal and minimal requirements of the animal and, hence, significant differences were noticeable in growth rate and in certain tissue constituents of the animals receiving the two supplements. However, no animal showed symptoms of avitaminosis through a period of 8 to 10 weeks. While, therefore, the greater food consumption of animals receiving the higher vitamin supplement can be attributed to a general improvement in these animals, this cannot be correlated specifically with any single vitamin deficiency *per se*.

The observations of Larson and Chaikoff ('37) and Munro ('49) indicate that deterioration in nitrogen retention as a result of separation of the protein and carbohydrate moieties of a purified diet is transitory and that very soon the animals reach a state of equilibrium. However, Geiger et al. ('50), using protein-depleted rats found that the differences in nitrogen utili-

zation persisted during a 14-day period. The present work confirms these findings.

Cuthbertson and Munro ('39) noted that the association of a small fraction of dietary nitrogen with the carbohydrate is sufficient to maintain nitrogen equilibrium in humans. In an extension of this work, Munro and Wikramanayake ('54) concluded that protein utilization is influenced favorably by some carbohydrate in the protein-containing meals. The present work with the rice-legume diets suggest that with marginal intake of vitamins, any sort of non-uniformity or irregularity in the daily diet will adversely affect protein utilization. This appears to be so even with intermittent feeding of high- and low-protein dietaries (Geiger et al., '55; Anna, Dam-Bakker, De Groot and Luyken, '58), apparent when two deficient proteins, which in combination, form a good protein mixture, or a deficient protein and the lacking amino acid, are administered intermittently (Henry and Kon, '46; Geiger, '47, '48).

Even in such cases of imbalance, higher levels of dietary B vitamins may to some extent bring about better protein utilization, as shown. This is attributable in part, perhaps, to a more balanced selection of food components by the animal. Richter and Hawkes ('41) showed, for example, that rats select varying proportions of protein, fat and carbohydrate when supplied with different B vitamins, presumably adjusting the intake to actual needs.

The favorable influence of carbohydrate association on nitrogen retention is not related to their caloric function, as this property is not shared by fats (Thomson and Munro, '55). In other work,[2] deterioration in nitrogen retention caused by substitution of fat for dietary carbohydrate calls for altered requirement of certain of the B vitamins has been observed. Obviously, there is a good deal of flexibility in nutritional needs based upon the stresses to which the organism is exposed (Elvehjem and Krehl, '55).

SUMMARY

Adult protein-starved rats were used to compare the influence of marginal and optimal intakes of the B vitamins (thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, biotin, folic acid, vitamin B_{12}, choline and inositol) on protein utilization from diets with uneven distribution of protein and caloric moieties or with intermittently changing levels of protein.

When the protein and caloric components of a purified 20% casein diet were fed separately with a 6-hour intervening period each day, a lowering in nitrogen retention resulted as well as efficiency of protein utilization when compared to the effect of the composite diet. This effect, persisting over a three-week period, was greater in the groups receiving minimal levels of B vitamins and was somewhat offset by the inclusion of optimal amounts of B vitamins in the diet. These changes were noted in studies with animals fed either ad libitum or with restriction.

A similar effect due to B vitamins was observed in split feeding of the major protein and carbohydrate moieties of a mixed rice-legume diet at a 15% level of protein.

With weanling rats, intermittent feeding on successive days of an 18% protein ration based on rice, a legume and casein and a 6% rice-protein ration lowered the protein efficiency when compared with that observed with a uniform intake of a 14% protein diet with rice, legume and casein. This effect, again, was a little more prominent when the intake of B vitamins was minimal. The beneficial effects due to B vitamins were associated with a decrease in the non-protein content of liver and plasma and a reduction in liver lipids.

[2] Fatterpaker and Sreenivasan, unpublished data.

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Effects of Suboptimal and Optimal Intakes of B Vitamins on Protein Utilization by the Growing Rat from Diets Containing Single and Mixed Proteins[1]

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It is recognized that vitamins may, to some extent, influence the minimal and optimal requirements of individual amino acids (Brock, '55). Sure and Romans ('48) and Sure ('50) observed an improvement in the protein efficiency of casein and fibrin with higher intakes of B vitamins. Nelson and Evans ('53) reported that impairment in the reproductive ability in rats on low-protein diets is partially corrected by doubling the vitamin supplements and the addition of vitamin B_{12}. In a previous publication (Marfatia and Sreenivasan, '60), it was demonstrated that, in the rat, B vitamins administered at optimal levels counteract the deterioration in nitrogen retention and growth rate arising from split feeding of the protein and carbohydrate moieties of the diet. The efficacy of higher dietary levels of B vitamins may be expected to differ with the over-all dietary protein quality and has now been ascertained, employing single- and mixed-protien diets derived from casein, egg albumin and wheat gluten and with variations in levels of thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid and vitamin B_{12}.

EXPERIMENTAL

Male albino rats of the Wistar strain were used. Prior to the experimental period these were reared on a balanced laboratory ration consisting of (percentages by weight): whole wheat flour, 75; casein, 12; whole milk powder, 2; yeast powder, 2; sodium chloride, 2; calcium carbonate, 2; arachis oil, 3; and shark liver oil, 2.

The composition of the experimental diets is given in table 1. Animals were housed individually in raised mesh-bottom cages and had free access to food and water.

Experiment 1. Trials with casein and egg albumin

In the first series, 32 rats with an average weight of 100 gm were used. The animals were divided into 4 comparable groups receiving diets containing purified egg albumin (diet A) or ethanol-extracted casein (diet B) as sole source of protein at the 10% level and supplemented with the B vitamins at one of two levels, a low sub-optimal and a high optimal. Growth was observed for a period of 5 weeks. At the end of this period the casein-fed animals were exsanguinated and the visceral organs collected and analyzed, individually and pooled, for total nitrogen and lipids.

In the second series of experiments, weanling rats, approximately 40 gm in weight, were similarly grouped and fed diets identical to the above except that the protein content was raised to the 18% level at the expense of starch. Growth was recorded at weekly intervals for a period of 6 weeks, at the end of which the animals were killed under ether anesthesia. Blood was collected from the hepatic portal vein, heparinized, and centrifuged in the cold to obtain the plasma. This was analyzed for total and non-protein nitrogen. Livers were quickly excised and chilled in cracked ice and determinations were made of total and non-protein nitrogen and total lipids.

Received for publication May 25, 1959.
[1] This investigation was supported by a research grant from the Williams-Waterman Fund, Research Corporation, New York.

TABLE 1
Composition of experimental diets[1]
(Quantities for 100 gm of diet)

Component	Experiment 1		Experiment 2		
	Diet A	Diet B	Diet C	Diet D	Diet E
	gm	*gm*	*gm*	*gm*	*gm*
Vitamin-free casein		10(or 18)			
Purified egg albumin[2]	10(or 18)		18	14	
Corn starch	80(or 72)	80(or 72)	72		
Whole wheat flour				76	76
Purified wheat gluten[2]					14
Vitaminized sucrose*	1	1	1	1	1
Vitaminized arachis oil†	5	5	5	5	5
Salt mixture, U.S.P. XIV	4	4	4	4	4

*B vitamins per gram sucrose

	Optimal	Sub-optimal
	mg	*mg*
Variable additions		
Thiamine·HCl	0.3	0.075
Riboflavin	0.4	0.1
Pyridoxine·HCl	0.3	0.075
Ca pantothenate	1.0	0.25
Niacin	2.0	0.5
Folic acid	0.1	0.025
Vitamin B_{12}	0.015	nil

Common additions	
	mg
Para-amino benzoic acid	10.0
Biotin	0.05
Inositol	20.0
Choline Cl	20.0
Menadione	0.5

† Fat-soluble vitamins per 5.0 gm arachis oil

	mg
Vitamin A acetate	0.31
Vitamin D (calciferol)	0.0045
Alpha tocopherol	5.0

[1] Each of the diets was modified further with additions in sucrose of 7 of the B vitamins either at optimal or at sub-optimal levels as indicated.

[2] Egg albumin and wheat gluten, Nutritional Biochemicals Corporation, Cleveland.

Experiment 2. Trials with wheat, egg albumin and mixed dietaries

In this setup the effect of two levels of B vitamins on the utilization of a wheat diet (diet E) was studied in comparison with the effect of diets containing either egg albumin (diet C) or mixed proteins (diet D). The total protein was at the 18% level in all diets and in the wheat diet this was adjusted with purified wheat gluten.

Six groups of weanling, 40 gm rats were fed these diets supplemented, as in experiment 1, with the B vitamins either at an optimal or a sub-optimal level. Body-weight records were maintained throughout the experimental period of 8 weeks. The animals were decapitated at the end of this period and livers analyzed for total and non-protein nitrogen, total lipids, total riboflavin and flavin adenine dinucleotide (FAD), pyridine nucleotides, coenzyme A (CoA), folic acid and vitamin B_{12}.

METHODS

The organs were blotted and weighed immediately after they were excised. Suitable portions were later made into 20% homogenates with ice-cold distilled water using a Potter-Elvehjem glass homogenizer. Aliquots of the homogenates were used for subsequent determinations.

Protein-free filtrates of plasma were prepared according to the procedure of Hier and Bergeim ('45); those of liver were obtained using trichloroacetic acid for protein precipitation. Nitrogen in plasma and in tissue homogenates and their protein-

free preparations was determined, after liberation by microKjeldahl digestion, by direct Nesslerization as described by Umbreit ('46). The determination of total lipids in tissue homogenates was done as described by Marfatia and Sreenivasan ('60). FAD and total riboflavin in liver were determined fluorimetrically by the procedure of Bessey, Lowry and Love ('49). Differential determination in liver of oxidized (PN) and reduced (PNH) pyridine nucleotides was carried out fluorimetrically by the procedure outlined by Dianzani ('55). For the assay of liver CoA the method of Kaplan and Lipmann ('48) was followed. Vitamin B_{12} in liver was liberated with papain (25 mg/gm of fresh liver) in an overnight incubation under toluene in acetate buffer, pH 4.6. The liberated vitamin was assayed using *Euglena gracilis* as the test organism (Hoff-Jorgensen, '54). Folic acid was liberated by autolysis of liver homogenates in 0.1 M phosphate buffer at pH 7.2 and was determined by the assay procedure of Mitbander and Sreenivasan ('54) using *Streptococcus faecalis R.*

RESULTS AND DISCUSSION

Utilization of casein and egg albumin

At the 10% level of protein, the growth rate with egg albumin was, as may be expected, consistently higher than with casein throughout the 5-week period, irrespective of the level of B vitamins in the diet. The average weight gains at the end of 5 weeks at sub-optimal and optimal intakes of the B vitamins were respectively 28 and 62 gm with casein diets and 68 and 78 gm with egg albumin diets. The growth response to B vitamins was thus considerably greater with casein diets in comparison with that obtained with diets containing egg albumin, so that differences in the growth rate due to type of protein narrowed with optimal intake of the vitamins.

In a similar study, Sure and Romans ('48) found that growth of rats on a low-casein (7.1%) diet was possible only when the various components of the vitamin B complex were raised to high concentrations and that with low intakes of the vitamins only maintenance could be achieved; the weight gained by the animals receiving the higher amounts of the B vitamins was mainly fat rather than protein. Sarett and Perlzweig ('43) also reported that, on low-protein diets, supplementation with B vitamins increased liver and body fat. The growth-promoting effect of vitamin B_{12} in rats deficient in this vitamin has also been attributed to increased lipogenesis (Black and Bratzler, '52; Knoebel and Black, '52; Ling and Chow, '52) which possibly results from increased choline synthesis (Arnstein, '55; Henry and Kon, '56). Similar increases in fat deposition have been observed on supplementing low quality protein diets with other lipotropic factors, methionine and choline (Shils, De Giovanni and Stewart, '55). In the present study, therefore, it was of interest to examine the composition of the weight gained by the casein-fed animals when the intake of the B vitamins was raised to optimal.

The data obtained from the analysis of the viscera of the casein-fed animals are presented in table 2. The results obtained from a pool of the different organs compare favorably with those obtained from individual determinations.

In all organs examined, an increase in tissue weight was evident on the low-vitamin diet. This effect was marked in the lung tissue and was least in the spleen. A striking increase in the nitrogen content of the pooled viscera and in individual tissues, with the high-vitamin diet, is, however, indicative of a more efficient feed utilization. However, in all these tissues a concomittant rise in lipids was also observed.

The growth data obtained when the dietary protein level was raised to 18% showed trends analogous to those observed with the 10% protein diets. Here, however, the growth rates with casein and egg albumin diets at optimal vitamin intake almost overlapped during the initial 5 weeks and significant differences due to protein type were apparent only at the end of the 6th week. Thus the average gains at the end of the 6-week experimental period with sub-optimal and optimal intakes of B vitamins were respectively 74 and 110 gm with casein diets and 95 and 126 gm with egg albumin diets.

TABLE 2

Effect of the level of dietary B vitamins on the visceral composition[1] of rats fed a 10% casein diet
(Experiment 1)

Tissue	Fresh organ weight		Tissue constituents			
			Total nitrogen		Total lipids	
	B vitamin supplement		B vitamin supplement		B vitamin supplement	
	Sub-optimal level	Optimal level	Sub-optimal level	Optimal level	Sub-optimal level	Optimal level
	gm	*gm*	*mg/gm fresh weight*		*mg/gm fresh weight*	
Heart	0.58 ± 0.04	0.55 ± 0.03	24.6 ± 1.5	32.2 ± 1.0	61.4 ± 5.1	67.6 ± 1.6
Lung	1.33 ± 0.05	0.99 ± 0.06	16.1 ± 0.7	23.7 ± 1.1	49.9 ± 4.1	83.1 ± 1.7
Liver	5.55 ± 0.58	5.35 ± 0.29	26.3 ± 1.4	37.4 ± 0.6	69.1 ± 2.2	78.0 ± 8.0
Spleen	0.38 ± 0.03	0.47 ± 0.03	24.6 ± 0.4	30.6 ± 0.4	70.9 ± 2.7	84.7 ± 2.8
Kidney	1.41 ± 0.05	1.17 ± 0.06	26.3 ± 1.5	35.8 ± 1.3	70.6 ± 2.8	83.1 ± 2.2
Adrenal	0.041 ± 0.006	0.031 ± 0.005	26.9 ± 0.6	34.9 ± 1.7	—	—
Muscle			25.1 ± 2.1	29.9 ± 0.8	39.3 ± 3.7	41.5 ± 3.6
Pooled viscera[2]			24.1 ± 0.6	31.9 ± 0.6	64.8 ± 2.5	79.2 ± 3.1

[1] Figures represent, in each case, the mean value from at least 5 replicates ± standard error of the mean.
[2] Pooled viscera included equal parts by weight of heart, lung, liver, spleen and kidney.

TABLE 3

Effects of the level of dietary B vitamins on certain liver and plasma constituents of rats fed diets with casein or egg albumin at the 18% level
(Experiment 1)

Protein	B vitamin supplement[1]	Liver			Plasma nitrogen	
		Total nitrogen	Non-protein nitrogen	Total lipids	Total	Non-protein
		mg/gm fresh weight			*mg/100 ml*	
Casein	Sub-optimal (8)	22.2 ± 1.2[2]	2.1 ± 0.3	101.0 ± 4.3	880 ± 11	81.2 ± 3.7
Casein	Optimal (7)	27.6 ± 1.6	1.7 ± 0.3	92.6 ± 3.4	1068 ± 22	49.1 ± 4.3
Egg albumin	Sub-optimal (8)	24.1 ± 0.9	1.9 ± 0.4	96.3 ± 5.1	962 ± 31	63.3 ± 5.1
Egg albumin	Optimal (7)	25.2 ± 1.5	1.8 ± 0.2	90.1 ± 3.2	1035 ± 26	42.9 ± 3.0

[1] Figures within parentheses represent the number of animals used in each experiment.
[2] Mean value ± standard error of mean.

Data obtained from liver and plasma analyses in these animals (table 3) reflect the composition of the growth. An increase in plasma protein concentration and a decrease in its non-protein nitrogen content resulted from a higher intake of B vitamins with either casein or egg albumin diets. These effects are suggestive of improved utilization of dietary amino acids. A similar trend could be seen in the data for liver nitrogen. There was also a striking modification of the effect of the B vitamins on liver fat content observed with the groups fed the 10% casein diet. On the other hand, in rats fed egg albumin there was no appreciable change in liver composition due to B vitamins.

Utilization of wheat gluten, egg albumin and mixed protein dietaries

Considerable improvement in the growth rate of rats fed the wheat gluten diet was noted as a result of supplementation with the higher level of the B vitamins. A partial to complete replacement of the wheat protein by egg albumin caused a corresponding decrease in growth response to the vitamins. The growth data and liver contents of nitrogen, lipids and sulfhydryl compounds are presented in table 4. There was no significant change in liver lipid content with increased intake of B vitamins but there was definite improvement in liver protein-nitrogen, especially with the wheat gluten diet.

The data on liver contents of vitamins and cofactors are presented in table 5. Although the increased retention of the vitamins and cofactors in the liver tissue with higher intake of the B vitamins was to be expected, it was observed that even an improvement in the average quality of dietary protein increased the retention of these growth factors except in the case of CoA which showed a reverse trend. The dependence of liver levels of certain vitamins on dietary protein was also demonstrated by Sarett and Perlzweig ('43), who observed that rats receiving a diet low in protein were unable to incorporate both nicotinic acid and riboflavin into the liver, even when a liberal intake of B vitamins was provided. Guggenheim, Halevy, Neumann and Usieli ('56) reported that low-protein and protein-free diets lower the liver contents of citrovorum factor (CF) and folic acid (PGA) and depress the ability of the tissue to convert PGA into CF in rats. The decrease in liver CoA as a result of improvement in the quality of dietary protein could apparently have been due to alterations in liver level of vitamin B_{12}. An inverse relationship between the CoA content of the liver and the vitamin B_{12} status of the organism has, thus, been demonstrated in chicks (Boxer, Ott and Shonk, '53) and in rats (Boxer, Shonk, Gilfillan, Emerson and Oginsky, '55; Wong and Schweigert, '56). The difference due to dietary protein quality levelled off at

TABLE 4

Effects of the level of dietary B vitamins on the liver composition of rats fed diets derived from wheat and egg albumin

(Experiment 2)

Source of protein	Level of B vitamins	Weight gain in 8 weeks	Liver: Total nitrogen	Liver: Non-protein nitrogen	Liver: Total lipids
		gm	*mg/gm fresh weight*		
Wheat flour and wheat gluten	Sub-optimal	90.2 ± 4.3[1] (10)[2]	19.6 ± 2.0	2.3 ± 0.2	74.6 ± 4.3
(18% protein)	Optimal	128.9 ± 6.6 (8)	29.9 ± 1.3	2.1 ± 0.3	69.9 ± 3.1
Wheat flour and egg albumin	Sub-optimal	152.8 ± 5.2 (10)	30.1 ± 2.3	2.1 ± 0.2	77.8 ± 5.1
(18% protein)	Optimal	167.0 ± 3.4 (9)	35.3 ± 1.9	3.1 ± 0.3	79.0 ± 2.2
Egg albumin	Sub-optimal	170.6 ± 4.8 (9)	34.6 ± 3.8	3.0 ± 0.6	79.2 ± 3.7
(18% protein)	Optimal	180.2 ± 7.2 (8)	35.6 ± 1.4	3.2 ± 0.2	81.1 ± 4.0

[1] Mean value ± standard error of mean.
[2] Figures within parentheses indicate the number of animals in each group.

TABLE 5

Liver contents[1] of riboflavin, pyridine nucleotides, coenzyme A, folic acid and vitamin B_{12} in rats fed diets derived from wheat and egg albumin
(Experiment 2)

Diet description		Riboflavin		Pyridine nucleotides		Co A	PGA	Vitamin B_{12}
Source of protein	Level of B vitamins	Total	FAD	PN	PNH			
		μg/gm	μg/gm	μg/gm	μg/gm	units/gm	μg/gm	mμ/gm
Wheat flour and wheat gluten	Sub-optimal	20.5 ± 1.2[2]	14.8 ± 0.8	559 ± 35	170 ± 18	213 ± 12	1.43 ± 0.32	28.9 ± 4.3
	Optimal	27.1 ± 1.0	24.7 ± 1.1	883 ± 21	313 ± 21	243 ± 11	1.87 ± 0.30	37.3 ± 3.8
Wheat flour and egg albumin	Sub-optimal	27.5 ± 2.7	22.7 ± 4.5	816 ± 59	311 ± 27	161 ± 10	1.71 ± 0.38	52.5 ± 4.9
	Optimal	30.8 ± 1.2	26.2 ± 1.5	946 ± 61	261 ± 14	213 ± 17	2.32 ± 0.28	69.9 ± 7.3
Egg albumin	Sub-optimal	26.9 ± 2.1	24.8 ± 3.1	745 ± 20	253 ± 10	145 ± 9	1.70 ± 0.21	81.9 ± 3.2
	Optimal	28.5 ± 1.3	28.0 ± 1.4	845 ± 63	261 ± 9	196 ± 13	2.04 ± 0.36	105.7 ± 12.1

[1] All values are expressed on fresh weight of the tissue.

[2] Mean value derived from at least 7 independent determinations ± standard error of mean.

the higher intake of the B vitamins. Under the latter conditions there was a relatively greater increase in FAD than in free riboflavin and also in PNH in comparison with PN.

Although the lower level of vitamins used in these experiments is sub-optimal, gross deficiency symptoms of any of the B vitamins were not observed. That the vitamins at the lower level were limiting was indicated by the improved growth obtained with the use of adequate protein and higher levels of B vitamins.

There seems to exist an inverse relationship between protein quality and the effectiveness with which B vitamins can be used at higher levels. With good quality or high-protein diets, the weight gains due to B vitamins are, perhaps, largely protein. On the other hand, the effects on growth of improved levels of B vitamins with inadequate protein dietaries may in part arise from increased lipogenesis. The improvements in protein utilization due to B vitamins may also be linked to the increased retention of the vitamins in the form of their cofactors with the use of high vitamin diets.

SUMMARY

Sub-optimal and optimal concentrations of 7 B vitamins (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid and vitamin B_{12}) have been used in purified diets in two series of experiments with the albino rat, one with casein and egg albumin, and the second, with wheat gluten, egg albumin and a mixture of the two serving as protein sources at levels of 10 or 18%.

With diets containing 10 or 18 % of casein and egg albumin, an increase in the levels of the B vitamins reduced the differences in growth rate due to protein quality. An examination of the viscera of animals fed the 10% casein diet revealed that the enhanced growth was the result of increased synthesis of both protein and lipid, although individual organs had decreased in weight.

With an 18% wheat gluten diet, a partial to total replacement of the protein by egg albumin resulted in progressive dimunition of growth promotion by the higher levels of the B vitamins.

On the egg albumin or mixed protein diets at the 18% level, B vitamins at the higher level increased plasma and liver protein and promoted increased retention of vitamins and cofactors in the liver tissue.

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Secretion of Labeled Blood Lipids into the Intestine[1]

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In studies of fat absorption based upon the recovery of lipids from the gastrointestinal tract, the question has arisen whether an endogenous secretion of lipid into the gut occurs during the process of absorption. A decrease in the specific activity of an orally administered lipid mixture, containing a free fatty acid as the label, has been observed in numerous studies (Borgstrom, '52a, b, '54; Ahrens and Borgstrom, '56a, b; Tidwell et al., '56; Burr et al., '59). Such a decrease would be compatible with the concept of a secretion of lipid into the tract during the process of absorption; however, this may likewise be explained by a preferential absorption of the labeled free fatty acids.

Ahrens and Borgstrom ('56a, b) have stated that a preferential absorption of the fatty acid takes place, and based on fatty acid analysis they have concluded that endogenous secretion of lipid need not occur to explain their results. In a study reported from this laboratory (Burr et al., '59) involving a doubly labeled lipid meal, supporting evidence for preferential absorption was obtained; however, the results suggested that a dilution with endogenous lipid might also be taking place.

As early as 1892, Voit interpreted the demonstration of neutral-fat, free fatty acids and soaps in prepared Thiry-Vella fistulas as representing secretion of lipid into the small intestine. Much later, Angevine ('29) measured the excretion of fat into a Thiry-Vella fistula in dogs maintained on different diets. In all cases, lipid secretion was demonstrated. In an analogous study employing iodine-labeled fat, Peretti ('35) recovered iodized fat from the Thiry-Vella fistula. From observations following a different experimental approach, Sperry and Bloor ('24) concluded that fecal fat may result from the secretion of blood fat.

The possibility of such a lipid secretion having a nutritional significance warrants further investigation of this problem. In order to study this question in the intact animal, labeled fat emulsions, labeled chyle and an albumin fatty acid complex of a labeled fatty acid were administered intravenously to rats, and the amount of labeled material appearing in the gastrointestinal tract was measured.

EXPERIMENTAL

White rats received only 50% of glucose and water for 48 hours. A polyethylene cannula was then introduced into the external iliac vein and extended into the inferior vena cava. The cannula was maintained patent by a very slow infusion of saline followed by 5% of glucose until the introduction of the labeled-fat sample was commenced 24 hours post-operatively. Immediately before starting the lipid infusion, 0.5 ml of olive oil was given each rat by feeding needle. In each case, the labeled lipid was introduced at a constant rate of about 1.6 ml per hour with the aid of a cam-driven syringe.

By maintaining the same infusion rate during a period of two and one-half to three hours, it was hoped that in the last hour of the experiment a nearly constant blood specific activity could be obtained. During the experiment, as previously de-

Received for publication July 31, 1959.

[1] This investigation was supported in part by grants from the Division of Research and Development, Office of the Surgeon General, Contract no. DA-49-007-MD-662, the Robert A. Welch Foundation, Houston, Texas, and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

scribed (Burr et al., '56), the rats were housed in a small glass cage, closed at one end with a rubber membrane through which the tail of the rat was allowed to protrude. This apparatus permitted continuous collection of the respiratory CO_2 and still allowed access to the tail vein for collection of blood. The polyethylene cannula was likewise brought out through the membrane.

Air freed of CO_2 was drawn through the cage, and the respiratory CO_2 collected in NaOH and precipitated as $BaCO_3$. The carbonate was dried overnight at 110°C and plated at infinite thickness in aluminum planchets for determination of radioactivity. The CO_2 and blood samples were collected periodically after the first hour of infusion. Blood was obtained from the tail vein and plated directly on aluminum planchets, using a porous support of lens tissue as previously described (Burr et al., '54). At the termination of the experiment, a cardiac puncture was performed under light ether anesthesia and the rat was exsanguinated. The small intestine from the pylorus to the cecum was removed and flushed with 100 ml of ether. The blood lipids were extracted with alcohol and ether and the residue from this extraction was saponified with alcoholic KOH, acidified and extracted with ether. The extracts were combined, dried and redissolved in petroleum ether. The lipid of the intestinal content was prepared as previously described (Deuel et al., '40; Tidwell et al., '56).

The labeled chyle employed in this study was obtained from the cannulated thoracic duct of a white rat which had been given orally a lipid meal labeled with palmitic acid-1-C^{14}. The triglyceride emulsion was prepared by dissolving the tripalmitin carboxyl-C^{14} in corn oil which contained 5% of purified egg lecithin. Approximately 25 mg of this lipid mixture was dissolved in 2 ml of ether and the solution homogenized with 25 ml of 5% dextrose solution in a tissue grinder with a Teflon pestle. During the process, the temperature of the mixture was gradually elevated to 50°C. The remaining ether was removed under reduced pressure at this temperature. Homogenization was continued until 90% or more of the lipid particles were 0.5 micron or less in diameter. The fatty acid albumin complex was prepared from palmitic acid-1-C^{14} and bovine albumin in a manner previously described (Johnston, '58). All samples of lipids were oxidized in a dry combustion apparatus and the millimoles of carbon determined manometrically (Burr et al., '59). The CO_2 was trapped in NaOH and precipitated as $BaCO_3$. Measurement of carbon 14 was made with a micromil-thin window counter.

RESULTS AND DISCUSSION

By a constant infusion of the labeled materials, a point should be reached at which the rate of entry and the rate of removal of the labeled material would be such that a relatively constant blood spe-

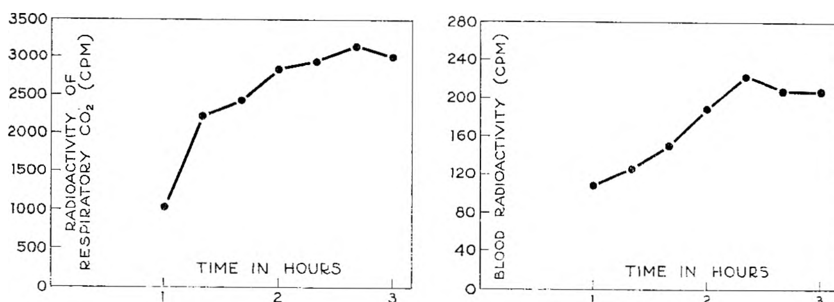

Fig. 1 Radioactivity of respiratory CO_2 and dried blood samples obtained from a rat receiving intravenously an emulsion containing C^{14}-labeled triglyceride. Blood samples are mounted on lens tissue and corrected for background and to a thickness of 1.4 mg/cm². All respiratory CO_2 samples are plated as $BaCO_3$ at infinite thickness and corrected for background.

cific activity could be maintained. Figure 1 shows a representative graph of the radioactivity of the blood at 20-minute intervals following the first hour of infusion of the triglyceride emulsion in one of the animals. Over the last hour the variation between values was not great. A complete sampling of blood was not carried out in each experiment. As an auxiliary measure, the respiratory CO_2 was collected and its specific activity assayed. Also shown in the figure are the activities of the respiratory CO_2 for the same animal. During the last hour, the deviation in specific activity of the respiratory CO_2 samples from their means varied only from 1 to 6.5% for all of the animals. The blood lipid level and the rate of the blood lipid undergoing oxidation roughly paralleled each other.

Animals were fed olive oil immediately prior to the infusion of the labeled lipid for two reasons: (1) this exogenous lipid aids as a trapping material if labeled lipid is secreted into the intestine and (2) an appreciable secretion might be associated with the absorptive process. The possibility was considered that dilution with the endogenous lipids might have nutritional significance in modifying the lipid being absorbed to resemble more closely that of the animal or, in some other manner, enhancing lipid absorption or utilization. Hoagland and Snider ('43) and Cheng et al., ('49) have established that the absorption of a fat such as tripalmitin is greatly increased by mixing it with an easily digestible lipid such as olive oil. Other investigators have suggested that lipids are altered in the lumen of the intestine (Sammons et al., '56). The possibility exists that some of these changes might be accounted for by the secretion of endogenous lipids.

In table 1 are shown the levels of radioactivity of the infused lipid, the blood lipid at the termination of the experiment and the intestinal lipid as well as the total activity in the intestinal lipid. If the assumption is made that any lipid secreted into the gut would have its origin in the blood lipid and if, in addition, it is further assumed that the specific activity of the blood lipid existing at the termination of the experiment is representative of this for a period of time, then it is possible to calculate the amount of this blood lipid which would have to be secreted to account for the total activity in the small intestine. These calculations are subject to the error of possible losses in the recovery of lipid from the intestine; also the possibility of a relatively rapid turnover must be considered, since much of the activity may have been reabsorbed. However, when such calculations are made, it appears that up to 11% of the intestinal lipid had its origin in the blood fat. This is probably a minimal measure of secretion since the labeled lipid may be rapidly reabsorbed and the percentage obtained re-

TABLE 1

Radioactivity[1] of infused and recovered lipids

	Infused lipid	Terminal blood lipid	Intestinal lipid recovered	Intestinal lipid	Intestinal lipid total cpm	Blood lipid equivalent to total activity of intestinal lipid	Intestinal lipid from blood lipid
	cpm/ml $\times 10^4$	*cpm/mg*	*mg*	*cpm/mg*	*cpm*	*mg*	*%*
Triglyceride[2]	6.6	86.8	192	1.1	202	2.3	1.3
Triglyceride[3]	3.2	9.1	447	0.57	256	28	6.3
Chyle[3]	1.5	8.6	270	0.35	95	11	4.1
Fatty acid albumin[2]	1.2	6.0	450	0.66	296	50	11.1
Fatty acid albumin[3]	2.3	7.4	231	0.68	156	21	9.1

[1] All samples counted as $BaCO_3$ at infinite thickness and corrected for background and expressed in counts per minute (cpm).

[2] Infusion period was three hours.

[3] Infusion period was 2½ hours.

presents only the amount present at that moment. Also, it is possible that the above assumptions are not completely valid and that the blood lipid may enter a lipid pool in the intestinal wall prior to its secretion into the gut. If this were the case, then the level of activity of the lipid recovered from the lumen of the intestine would not be an accurate measure of total secretion since it would not include the unlabeled lipid from the lipid pool of the intestinal wall. The values obtained in this experiment are considerably less than those based upon the loss in specific activity within the tract (Burr et al., '59). Another explanation of the differences obtained by these two experimental approaches would be that a system exists in which a portion of the absorbed fat mixes with that of the intestinal cells and is then resecreted into the gut without the necessity of first entering the blood stream. Such a secretion has been suggested by Johnston ('59).

The three different types of infused lipids were studied to determine whether the secretion might be dependent upon the type of circulating lipid available. While the greatest values were obtained with the fatty acid albumin complex, there is not a sufficient number of animals to warrant any definite conclusion.

The data support the hypothesis that some lipid re-enters the intestinal tract but suggest that if the blood fat is the immediate source of this secretion, then quantitatively, it is less significant than would be indicated by studies based on the decrease in specific activity of a fed labeled lipid.

SUMMARY

Labeled-fat emulsions, labeled chyle and an albumin fatty acid complex of a labeled fatty acid were administered intravenously to rats over a two and one-half or three-hour period. To determine the amount of blood lipid secreted into the gastrointestinal tract, the animals were killed and the lipid isolated from the gastrointestinal content. Based on the radioactivity of the recovered lipid, it was calculated that up to 11% of the intestinal lipid resulted from the labeled lipid administered intravenously. The relationships of the reported observation to the overall secretion of lipid material into the intestinal lumen is discussed.

ACKNOWLEDGMENT

The authors gratefully acknowledge the technical assistance of Mrs. Barbara Mollenhauer, Malcolm Hammett and Roy Askins.

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Supplementation of Cereal Proteins with Amino Acids

III. EFFECT OF AMINO ACID SUPPLEMENTATION OF WHEAT FLOUR AS MEASURED BY NITROGEN RETENTION OF YOUNG CHILDREN[1,2]

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Previous work published by the Institute of Nutrition of Central America and Panama (INCAP) on the effect of amino acid supplementation of corn masa (Bressani et al., '58c; Scrimshaw et al., '58) demonstrated that the biological value of corn protein as measured by nitrogen balance in children could be increased by the simultaneous addition of lysine and tryptophan and by the further addition of isoleucine. Recently, Truswell and Brock ('59) confirmed these results using adult subjects. The studies of amino acid supplementation of corn protein were undertaken in part to test the usefulness of the amino acid proportions of the FAO "reference protein" ('57). This "reference protein" represents the initial attempt of an Expert Committee on Protein Requirements of the Food and Agriculture Organization of the United Nations (FAO) to devise an optimal amino acid pattern for human growth and maintenance based on the composition of proteins of known high biological value, and on the various studies of amino acid requirements in man and experimental animals.

To obtain further knowledge of the applicability of this proposed amino acid pattern, studies of the effect of amino acid supplementation of other proteins are required. Because of the importance of wheat in human nutrition, this was selected as the next staple food to be studied. The many reports based on studies in experimental animals, which indicate that the biological value of wheat flour can be significantly improved by the addition of lysine, have been summarized recently by Rosenberg and Rohdenburg ('52), Flodin ('53, '56), Rosenberg ('59) and Harris and Burress ('59). The present paper describes the results of the supplementation of wheat flour with essential amino acids in the proportions of the FAO "reference protein" ('57). The observations were made on hospitalized children who had recovered from severe protein malnutrition.

MATERIALS AND METHODS

Techniques described previously (Bressani et al., '58c; Scrimshaw et al., '58) were used to measure the nitrogen balance in 6 boys in 8 experiments involving, in most cases, two-day adaptation periods followed by three three-day balance periods with each diet combination. The age and weight of the children at the start of the experiments were as follows:

Case no.	Experiment no.	Age	kg
PC-83	1	2 years, 2 months	9.2
	2	2 years, 4 months	10.5
PC-88	1	3 years, 1 month	10.6
	2	3 years, 5 months	12.7
PC-89		1 year, 5 months	7.7
PC-91		5 years, 9 months	11.9
PC-92		2 years, 11 months	10.3
PC-97		3 years, 3 months	12.6

In preliminary feeding trials, a basal diet containing 5% of wheat gluten and

Received for publication August 8, 1959.

[1] Assisted by grants-in-aid from the National Millers' Federation, Washington, D. C., and from E. I. du Pont de Nemours and Company, Wilmington, Delaware.

[2] INCAP Publication I-142.

2% of L-glutamic acid, with the same amounts of the other ingredients, was used. This basal diet was, however, refused by the children because of the unpleasant flavor of L-glutamic acid. This was, therefore, omitted from the basal diet and the level of wheat gluten increased to 7%. Accordingly, the basal diet used in all experiments contained, in grams per 100 gm: wheat flour,[3] 85; wheat gluten,[4] 7; glycine, 3; and corn starch, 5. Also a vitamin and mineral capsule[5] was given daily. Nitrogen content of the basal diet was 3%. The amino acids added were substituted for corn starch, and the nitrogen from these replaced glycine nitrogen so that all diets remained isocaloric and isonitrogenous. They were fed at the rate of 2 gm of protein/kg/day with caloric intakes of 80 to 100/kg/day, according to the estimated requirement of the child.

The essential amino acid content of the wheat flour, the wheat gluten, the basal diet, and the FAO "reference protein" is given in table 1. According to the pattern of the "reference protein," the order of deficiency in the basal diet from the greatest to the least limiting amino acids was as follows: lysine, tryptophan, methionine, isoleucine, valine and threonine, the three latter appearing limiting to an equal degree. Also shown in the table is the amount of each amino acid in milligrams per gram of nitrogen of the basal diet, needed to make up the difference between the reference amino acid level and the amount present in the basal wheat diet. The amino acids were added progressively to the basal diet in approximately these amounts. Because initial calculations of the amino acid content of the wheat flour–wheat gluten basal diet were based on values from the literature, the quantities added were very slightly different from the amounts shown in table 1, based on subsequent analyses by microbiological methods previously referred to (Bressani and Scrimshaw, '58). Corrections were made for the D-form of the amino acids used, by doubling the amount added, except for DL-methionine which was

[3] Wheat flour milled in Guatemala, having the following chemical composition in gm/100 gm: moisture, 14.0; nitrogen, 1.88; ether extract, 1.30; crude fiber, 0.40; ash, 0.58. This flour is prepared from a mixture of locally grown and imported wheat.

[4] Chemical composition in gm/100 gm: moisture, 6.0; nitrogen, 12.88; ether extract, 1.60; crude fiber, 0.60; ash, 0.84.

[5] VI-Syneral, courtesy of the U. S. Vitamin Corporation.

TABLE 1

Essential amino acid composition of the wheat flour, wheat gluten, the basal wheat diet and of the FAO "reference protein"

Amino acid	Wheat flour	Wheat gluten[1]	Basal diet	Basal diet	FAO "reference protein"	Adequacy of basal diet	Amount of amino acids to be added
	gm %	*gm %*	*gm %*	*mg/gm nitrogen*	*mg/gm nitrogen*	*%*	*mg/gm nitrogen of basal diet*
Arginine	0.430	3.481	0.610	203	—	—	—
Histidine	0.292	1.825	0.374	125	—	—	—
Isoleucine	0.450	3.677	0.640	213	270	79	57
Leucine	0.705	5.993	1.018	339	306	—	—
Lysine	0.299	1.530	0.361	120	270	44	150
Methionine	0.190	1.389	0.259				
				149	270	55	121
Cystine	0.210[1]	1.726	0.189				
Phenylalanine	0.610	4.351	0.827		180		
				377		—	—
Tyrosine	0.359[1]	2.596	0.305		180		
Threonine	0.345	2.119	0.441	147	180	82	33
Tryptophan	0.098	0.856	0.143	48	90	53	42
Valine	0.460	3.789	0.656	219	270	81	51

[1] Orr, M. L., and B. K. Watt. Amino Acid Content of Foods. Home Economics Research Report no. 4, U.S.D.A., Washington, D. C., December, 1957.

TABLE 2
Nitrogen balance results of case PC-83 fed wheat flour supplemented with amino acids

Diet fed	Weight change	Nitrogen						
		Intake	Fecal	Absorbed	Absorption	Urinary	Retained	Retention
	kg	*mg/kg/day*			%	*mg/kg/day*		%
Experiment 1								
Milk	9.16	320	77	243	76.0	186	57	18.0
Basal	9.47	345	47	298	86.4	257	41	11.7
Basal	9.47	310	39	271	87.5	248	23	7.6
Basal	9.55	295	31	264	89.4	261	3	0.8
Basal + 0.64% L-lysine·HCl	9.55	323	49	274	84.9	243	31	9.8
Basal + 0.64% L-lysine·HCl	9.55	324	27	297	91.7	249	48	14.8
Basal + 0.64% L-lysine + 0.97% DL-valine	9.72	331	34	297	89.6	202	95	28.6
Basal + 0.64% L-lysine + 0.97% DL-valine	9.72	309	38	271	87.7	189	82	26.5
Basal	10.09	279	36	243	87.1	216	27	9.7
Basal	10.34	311	38	273	88.0	211	62	20.1
Experiment 2								
Basal	10.49	323	35	288	89.0	220	68	21.1
Basal	10.49	327	40	287	87.9	263	24	7.6
Basal	10.49	327	38	289	88.5	279	10	3.4
Basal + 0.64% L-lysine·HCl	10.74	272	42	230	84.6	178	52	19.1
Basal + 0.64% L-lysine·HCl	10.74	340	38	302	88.8	202	100	29.3
Basal + 0.64% L-lysine·HCl	10.74	325	33	292	90.0	232	60	18.5
Basal + 0.64% L-lysine + 0.97% DL-valine	11.03	356	38	318	89.4	259	59	16.8
Basal + 0.64% L-lysine + 0.97% DL-valine	11.03	345	39	306	88.7	215	91	26.6

assumed to be fully utilized. The amount of lysine added was corrected for the hydrochloride molecule present in the form used.

RESULTS

Case PC-83. The results of two experiments are given in table 2 and summarized in figure 1. The child retained 18% of nitrogen when milk protein was fed, while with the basal diet, nitrogen retention decreased. Lysine added to the basal diet increased nitrogen retention. When the studies were initiated, the original FAO "reference protein" value of 360 mg/gm of total nitrogen was used as the reference level for valine. This was later changed to 270 mg on the basis of additional data available prior to publication of the report. The 360-mg value made valine the second limiting amino acid in wheat, and the effect of adding valine to the basal plus lysine diet was studied with PC-83. With the lysine plus valine diet the retention appeared to increase. When the child was again fed the basal diet, retention decreased to about 15%. In the second experiment, the lysine effect was more marked and valine had no further effect.

Cases PC-88 and PC-91. The values of the nitrogen balances, obtained with cases PC-88, experiment 1, and PC-91, are presented jointly in table 3 and summarized in figure 1. Nitrogen retention when fed the milk diet was 26% of the intake. Upon feeding the basal diet, nitrogen retentions decreased. The next diet consisted of the supplementation of the basal diet with lysine; addition of this amino acid caused an increase only in the second period.

The results of case PC-91 indicate that an average retention of 23% of the nitrogen in the form of milk was observed. The basal diet resulted in a decreased nitrogen retention. The addition of lysine to the basal diet increased retentions to an average of 22% of the intake. According to the FAO "reference protein," the next limit-

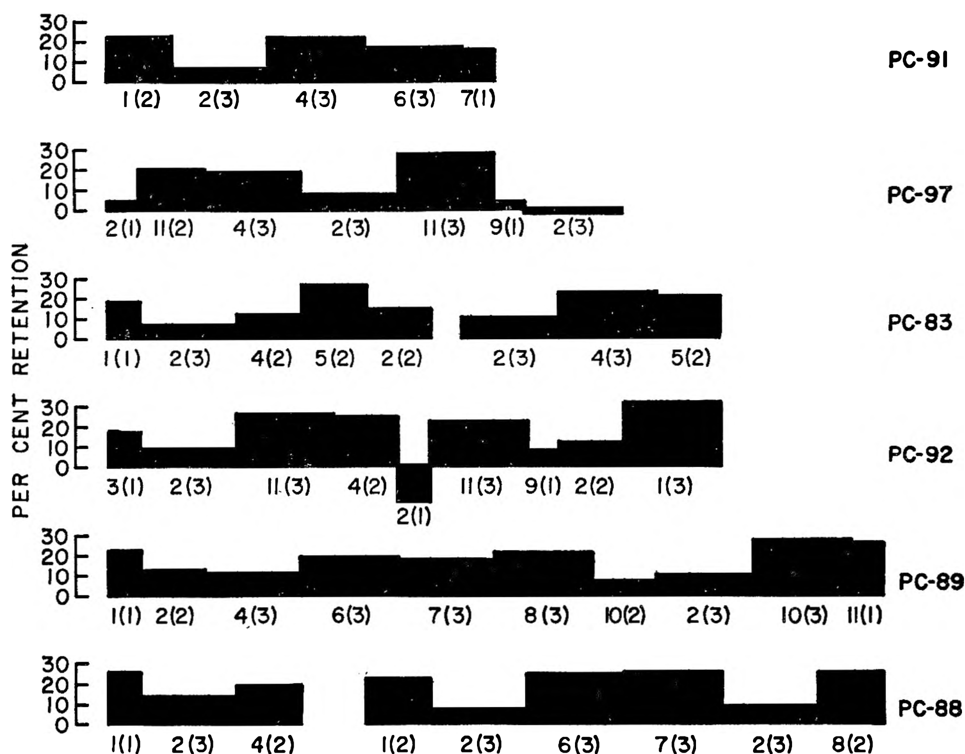

Fig. 1 Percentage of nitrogen retention of intake of children fed wheat diets supplemented with various essential amino acids.

Key to diets: The figures beneath the retention bars represent the diet fed, while those in parentheses represent the number of three-day balance periods. Amounts of amino acids added are listed in table 1.

1 Milk
2 Basal
3 INCAP vegetable mixture 9
4 Basal + lysine
5 Basal + lysine + valine
6 Basal + lysine + tryptophan
7 Basal + lysine + tryptophan + methionine
8 Basal + lysine + tryptophan + methionine + threonine
9 Basal + tryptophan + methionine + threonine + isoleucine + valine
10 Basal + lysine + tryptophan + methionine + threonine +isoleucine
11 Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine

ing amino acid after lysine was tryptophan, and the third, methionine. Addition of these two amino acids to the basal plus lysine diet did not alter retentions from those previously obtained with the basal wheat diet supplemented with lysine alone.

Case PC-88. The results of a second experiment are presented in table 4 and summarized in figure 1. The nitrogen retentions observed with milk protein were similar to those of the previous cases. The child was then fed the basal wheat diet, and retention decreased to an average of 8% of the intake. The next diet consisted of supplementing the basal diet with lysine and tryptophan. The results show that retentions increased to an average value of 25%. As in the previous cases, methionine addition to the basal diet plus lysine plus tryptophan did not alter nitrogen retentions. When the child was again fed the basal diet, retentions dropped to an average value of 12%. Upon feeding the basal diet supplemented with lysine, tryptophan, methionine and threonine, the child retained an average of 25% of the nitrogen intake,

TABLE 3

Nitrogen balance results of cases PC-88 and PC-91 fed wheat flour supplemented with amino acids

Diet fed	Weight change	Nitrogen						
		Intake	Fecal	Absorbed	Absorption	Urinary	Retained	Retention
	kg	*mg/kg/day*			%	*mg/kg/day*		%
Case PC-88								
Milk	10.6	321	69	252	78.5	169	83	26.0
Basal	11.1	329	50	279	84.8	248	31	9.4
Basal	11.1	324	61	263	81.2	214	49	15.1
Basal	10.9	326	39	287	87.9	228	59	17.7
Basal + 0.67% L-lysine·HCl	10.9	326	60	266	81.6	240	26	8.0
Basal + 0.67% L-lysine·HCl	10.9	350	51	299	85.4	198	101	28.9
Case PC-91								
Milk	11.9	292	64	228	78.2	148	80	27.6
Milk	11.9	288	61	227	78.9	174	53	18.5
Basal	12.3	349	38	311	89.2	279	32	9.2
Basal	12.3	339	69	270	79.7	276	−6	−1.8
Basal	12.3	316	23	293	92.9	249	44	14.1
Basal + 0.67% L-lysine·HCl	12.6	340	51	289	84.9	225	64	18.6
Basal + 0.67% L-lysine·HCl	12.6	327	35	292	89.2	187	105	32.0
Basal + 0.67% L-lysine·HCl	12.6	329	50	279	84.7	229	50	15.1
Basal + 0.67% L-lysine + 0.20% DL-tryptophan	12.9	321	36	285	88.8	227	58	18.0
Basal + 0.67% L-lysine + 0.20% DL-tryptophan	13.0	331	61	270	81.7	219	51	15.4
Basal + 0.67% L-lysine + 0.20% DL-tryptophan	13.0	344	60	284	82.6	225	59	17.2
Basal + 0.67% L-lysine + 0.20% DL-tryptophan + 0.35% DL-methionine	13.2	305	50	255	83.6	204	51	16.7

a retention which was similar to that obtained with lysine and tryptophan supplementation.

Case PC-89. As shown in table 5 and summarized in figure 1, feeding milk protein resulted in a retention of 23%. The basal diet produced a decrease in nitrogen retention to an average of 13%. The addition of lysine to the basal diet resulted in an average retention of 11%, and when this diet was further supplemented with tryptophan, retentions increased to an average of 20%. The basal diet was then supplemented with lysine, tryptophan and methionine, resulting in an average retention of 20% which is equal to that obtained when the basal diet was supplemented with lysine and tryptophan. The addition of threonine to the lysine, tryptophan and methionine–supplemented diet resulted in an average retention of 22%, and when this diet was supplemented with isoleucine, retention decreased to an average of 7%. The basal diet fed for the next period maintained retentions at an average of 10%, whereas feeding the basal diet supplemented with 5 and 6 of the limiting amino acids gave a retention of approximately 28%.

Case PC-97. As shown in table 6 and summarized in figure 1, feeding of the basal diet resulted in a low retention as in the previous cases. The basal wheat diet supplemented with all 6 of the limiting amino acids gave an average retention of 20%. When the child was fed the basal diet supplemented with lysine alone, retentions were as high as with the basal diet sup-

TABLE 4

Nitrogen balance results of case PC-88 fed wheat flour supplemented with amino acids in a second experiment

Diet fed	Weight change	Nitrogen						
		Intake	Fecal	Absorbed	Absorption	Urinary	Retained	Retention
	kg	*mg/kg/day*			*%*	*mg/kg/day*		*%*
Milk	12.7	314	60	254	80.8	166	88	27.8
Milk	12.7	312	59	253	81.1	194	59	18.9
Basal	12.8	333	41	292	87.7	263	29	8.8
Basal	12.8	346	47	299	86.4	267	32	9.1
Basal	12.8	323	40	283	87.7	268	15	4.7
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan	13.0	329	47	282	85.7	207	75	22.8
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan	13.0	328	29	299	91.2	212	87	26.4
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan	13.0	334	36	298	89.2	214	84	25.0
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan + 0.35% DL-methionine	13.4	327	38	289	88.4	217	72	22.0
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan + 0.35% DL-methionine	13.4	350	40	310	88.5	211	99	28.2
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan + 0.35% DL-methionine	13.4	344	41	303	88.2	211	92	26.7
Basal	13.8	305	46	259	84.9	233	26	8.3
Basal	13.8	338	42	296	87.6	253	43	12.7
Basal	13.8	294	38	256	87.1	243	13	4.4
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan + 0.35% DL-methionine + 0.32% DL-threonine	13.9	320	35	285	89.1	202	83	25.9
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan + 0.35% DL-methionine + 0.32% DL-threonine	13.9	324	36	288	88.9	210	78	24.1

plemented with all 6 of the limiting amino acids. Again the basal diet gave a low nitrogen retention of around 9% of the intake. Upon supplementation with all 6 amino acids, an average retention of 30% was observed. When lysine was omitted, nitrogen retention dropped to 4%. Continuation was not possible because the child persistently vomited in the next 6 days and balance data could not be obtained. He continued to do so even when fed the basal diet, with the result that nitrogen balances were negative.

Case PC-92. As shown in table 7 and summarized in figure 1, feeding INCAP Mixture 9, a combination of vegetable ingredients, containing 27.5% of protein of high biological value (Institute of Nutrition of Central America and Panama, '58), resulted in a retention of 18%. The basal diet gave an average retention of 9%, and when this was supplemented with all of

TABLE 5

Nitrogen balance results of case PC-89 fed wheat flour supplemented with amino acids

Diet fed	Weight change	Nitrogen						
		Intake	Fecal	Absorbed	Absorption	Urinary	Retained	Retention
	kg	*mg/kg/day*			*%*	*mg/kg/day*		*%*
Milk	7.7	318	61	257	80.9	186	71	22.2
Basal	8.1	269	28	241	89.7	207	34	12.8
Basal	8.1	337	39	248	88.4	250	48	14.4
Basal + 0.67% L-lysine·HCl	8.1	321	49	272	84.6	247	25	7.5
Basal + 0.67% L-lysine·HCl	8.1	343	34	309	90.1	248	61	17.6
Basal + 0.67% L-lysine·HCl	8.1	321	39	282	87.7	257	25	7.8
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan	8.6	358	39	319	89.2	242	77	21.5
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan	8.6	354	27	327	92.3	244	83	23.2
Basal + 0.67% L-lysine·HCl + 0.20% DL-tryptophan	8.6	347	34	313	90.2	267	46	13.5
Basal + lysine + tryptophan + 0.35% DL-methionine	8.8	343	39	304	88.7	266	38	11.1
Basal + lysine + tryptophan + 0.35% DL-methionine[1]	8.8	353	22	331	93.7	243	88	24.8
Basal + lysine + tryptophan + methionine + 0.32% DL-threonine	9.1	342	22	320	93.6	224	96	28.1
Basal + lysine + tryptophan + methionine + 0.32% DL-threonine	9.1	339	36	303	89.4	228	75	22.1
Basal + lysine + tryptophan + methionine + 0.32% DL-threonine	9.1	363	55	308	85.0	253	55	15.2
Basal + lysine + tryptophan + methionine + threonine + 0.43% DL-isoleucine[2]	9.4	320	53	267	83.4	241	26	8.1
Basal + lysine + tryptophan + methionine + threonine + 0.43% DL-isoleucine[2]	9.4	315	38	277	87.9	258	19	6.0
Basal	9.5	330	42	288	87.4	245	43	13.3
Basal	9.5	345	44	301	87.2	256	45	13.0
Basal	9.5	354	50	304	85.8	289	15	4.4
Basal + lysine + tryptophan + methionine + threonine + 0.43% isoleucine	9.9	373	43	330	88.6	225	105	28.3
Basal + lysine + tryptophan + methionine + threonine + 0.43% isoleucine	9.8	320	43	277	86.6	195	82	25.6
Basal + lysine + tryptophan + methionine + threonine + 0.43% isoleucine	9.8	349	64	285	81.8	187	98	28.2
Basal + lysine + tryptophan + methionine + threonine + isoleucine + 0.43% DL-valine	10.2	323	36	287	88.9	202	85	26.3

[1] Six-day balance period. [2] Vomiting.

TABLE 6

Nitrogen balance results of case PC-97 fed wheat flour supplemented with amino acids

Diet fed	Weight change	Nitrogen Intake	Fecal	Absorbed	Absorption	Urinary	Retained	Retention
	kg	*mg/kg/day*			*%*	*mg/kg/day*		*%*
Basal	12.6	348	49	299	86.0	281	18	5.3
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	12.7	362	39	323	89.2	243	80	22.1
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	12.7	349	54	295	84.5	231	64	18.3
Basal + L-lysine·HCl	13.1	374	61	313	83.8	241	72	19.2
Basal + L-lysine·HCl	13.1	393	57	336	85.6	249	87	22.1
Basal + L-lysine·HCl	13.1	376	56	320	85.1	254	66	17.6
Basal	13.6	361	92	269	74.6	277	−8	−2.1
Basal	13.6	338	48	290	85.8	234	56	16.7
Basal	13.6	348	54	294	84.4	254	40	11.3
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	13.8	368	56	312	84.9	180	132	35.4
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	13.8	369	59	310	84.0	200	110	29.8
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	13.8	370	56	314	84.8	226	88	23.6
Basal + tryptophan + methionine + threonine + isoleucine + valine[1]	14.2	321	64	257	80.1	243	14	4.3
Basal	14.5	318	64	254	79.9	256	−2	−0.6
Basal	14.5	270	61	209	77.4	240	−31	−11.5
Basal	14.5	332	67	265	79.9	264	0	0

[1] This diet was fed for two additional 3-day periods, but balances were not carried out due to excessive vomiting.

the amino acids deficient by comparison with the FAO "reference protein," the average retention was 27%. The next diet consisted of the basal wheat diet supplemented with lysine, which gave an average retention of 25%. Return to the basal diet resulted in a negative nitrogen retention, as a consequence of vomiting. When the basal diet was supplemented with all of the amino acids found limiting according to the FAO pattern, an average retention of 23% was observed. Retentions dropped to 9% when the basal wheat diet was supplemented with the 5 amino acids next limiting after lysine. The last diet consisted of feeding milk protein, and in this instance retentions were similar to those observed with the basal wheat diet supplemented with lysine alone or with all of the amino acids deficient by comparison with the FAO "reference protein."

TABLE 7

Nitrogen balance results of case PC-92 fed wheat flour supplemented with amino acids

Diet fed	Weight change	Nitrogen						
		Intake	Fecal	Absorbed	Absorption	Urinary	Retained	Retention
	kg	*mg/kg/day*			%	*mg/kg/day*		%
Vegetable mixture	10.3	330	96	234	70.9	174	60	18.2
Basal	10.5	341	48	293	85.9	263	30	8.8
Basal	10.5	364	55	309	85.0	271	38	10.6
Basal	10.5	347	51	296	85.3	270	26	7.5
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	10.9	365	50	315	86.4	204	111	30.5
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	11.0	333	57	276	82.8	211	65	19.4
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	11.0	346	61	285	82.3	175	110	31.9
Basal + lysine	11.6	346	55	291	84.0	205	86	24.9
Basal + lysine	11.6	345	51	294	85.1	207	87	25.0
Basal[1]	11.9	295	102	193	65.6	245	−52	−17.6
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	11.9	310	52	258	83.2	196	62	20.0
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	11.9	345	45	300	87.0	203	97	28.1
Basal + lysine + tryptophan + methionine + threonine + isoleucine + valine	11.9	343	52	291	84.8	217	74	21.6
Basal + tryptophan + methionine + threonine + isoleucine + valine[1]	12.6	253	42	211	83.5	189	22	8.6
Basal	12.3	332	43	289	87.0	230	59	17.8
Basal[1]	12.3	268	38	230	85.8	210	20	7.4
Milk	12.6	307	47	260	84.6	129	131	42.7
Milk	12.6	269	58	211	78.4	150	61	22.5
Milk	12.6	311	61	250	80.6	151	99	32.0

[1] Vomiting.

DISCUSSION

The nitrogen balance data presented show that the nutritive value of wheat proteins can be improved markedly by the addition of lysine alone to the wheat diet; in some cases the retention approximated that obtained with milk protein. Apparently, however, more consistent results are obtained if tryptophan is added together with lysine, and also with the addition of the other limiting amino acids according to the FAO "reference protein" levels. This result was to be expected, since the amount of added lysine exceeded that required to bring its relative proportion to that of tryptophan, the second limiting amino acid in wheat, according to the amino acid pattern of the reference protein. Therefore, lysine deficiency could have caused an imbalance which resulted in a decrease in nitrogen retention. Yang, Clark and Vail[6] have shown, in rats, that the mean growth rate, food and ntirogen intake, nitrogen efficiency ratio, nitrogen retention and bio-

[6] Yang, S. P., H. E. Clark and G. E. Vail 1959 Effect of level and method of lysine supplementation on the nutritive value of wheat proteins for young rats. Federation Proc., *18:* 553 (abstract).

logical value of a 10% protein wheat diet were at a maximum when supplemented with 0.20% of lysine and decreased when less or more lysine was added.

Amino acid supplementation is often less effective because the amount of amino acids added frequently is larger than actually needed and causes imbalances. As has been pointed out repeatedly by Rosenberg ('59), the biological value of cereal grains is improved by adding the amount necessary to bring the level of the first limiting amino acid to that of the second. He showed also that 90% wheat diets could be improved significantly by adding 0.3 to 0.4% lysine, respectively, to rations fed to female and male rats. The level of protein intake is probably also a very important factor in determining the optimal level of lysine or any other amino acid. This has been clearly shown in rats (Bressani and Mertz, '58; Harris and Burress, '59), chicks (Grau, '48) and swine (Becker et al., '57), using wheat, corn and other diets.

The importance of supplementing wheat protein with lysine, and the significance of its relation to the proportions of other essential amino acids, are particularly apparent from the results obtained in the last two cases, PC-97 and PC-92, since the feeding of the wheat basal diet supplemented with all the limiting amino acids according to the FAO "reference protein" gave nitrogen retentions similar to those obtained with the lysine supplementation alone. Omitting lysine from this diet, however, immediately resulted in vomiting and refusal of the food offered. This effect was probably due to the fact that the addition of other amino acids increased the imbalance of the diet with respect to lysine. Kumta et al. ('58) have shown that rats immediately refuse to consume imbalanced diets.

The amino acid pattern of the FAO "reference protein" or any other theoretical pattern of amino acids for the estimation of the order of amino acid deficiency in a test protein is very useful in interpreting the nitrogen balance results of the amino acid supplementation of food proteins. The results of supplementing wheat protein to meet the content of the FAO "reference protein" with respect to a given amino acid are quite different from those observed with the supplementation of corn protein in exactly the same manner. For example, comparison with the FAO pattern indicated that both corn masa and wheat proteins were deficient in methionine. Not only did the addition of methionine fail to bring about a positive or improved nitrogen balance in either study, but also its addition to corn masa protein resulted in a decreased nitrogen balance even when the basal diet was supplemented with lysine and tryptophan (Bressani et al., '58; Scrimshaw et al., '58). No such effect was observed in the studies with wheat.

In corn masa proteins, other amino acids besides lysine, tryptophan and isoleucine are apparently needed to counteract the effect of even a small excess of methionine, but in wheat protein these or other amino acids seem to be present in sufficient amounts so that a small excess of methionine has no effect. These results suggest that the 270 mg of methionine plus cystine in the FAO "reference protein" are too high.

The use of three three-day nitrogen balance periods to test the effect of the addition of the limiting amino acids to wheat has an advantage over the one and two three-day periods used in the previous studies. The results suggest that a good amino acid supplementation tends to produce a sustained response, while a poor or less satisfactory supplementation causes a negative balance or a transient increase in nitrogen retention. This is owing presumably to a temporary benefit from filling nitrogen needs with incomplete proteins which do not require the same amino acid proportions as overall growth and maintenance. A decrease in nitrogen retention following an initial rise due to an amino acid change suggests that the amino acid supplementation is inadequate. More extensive experiments are required to obtain the necessary data for quantitative evaluation of these phenomena.

SUMMARY

Six children recently recovered from severe protein malnutrition, ranging in age from one year, 5 months to 5 years, 9 months and in weight from 7.7 to 12.7 kg, were fed a simplified wheat diet in which the protein was contributed by both the

flour and gluten from wheat, in 8 experiments. All of the children were fed 2 gm of protein and 80 to 100 Cal./kg of body weight/day; a vitamin and mineral capsule was also given. The effect of the addition of the limiting amino acids according to the FAO "reference protein" was measured in most cases using three three-day balance periods. Comparison of the essential amino acid pattern of the wheat basal diet to the FAO "reference protein" showed that the order of the limiting amino acids was: lysine, tryptophan, methionine, isoleucine, valine and threonine. The results supply further evidence that in the utilization of wheat protein, lysine is the most limiting amino acid, since in most of the cases described in this paper, its addition to the basal wheat diet produced a sustained nitrogen retention sometimes similar to that obtained with milk feeding or observed when the basal diet was supplemented with all of the limiting amino acids according to the pattern of the FAO "reference protein." In one of two trials an improvement in nitrogen retention was obtained upon the addition of tryptophan to the lysine–supplemented diet. The refusal and vomiting of the diet fed and a reduction in nitrogen retention when the basal diet was supplemented with all the limiting amino acids except lysine, was further evidence of the importance of the lysine deficiency of wheat diets. For wheat protein, at least, the level of methionine in the FAO "reference protein" appeared to be too high since its addition did not improve nitrogen retention.

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The Requirement of the Baby Pig for Orally Administered Iron[1]

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The welfare of the baby pig is closely associated with its iron metabolism. Limited iron stores and low iron intake predispose the animal to the development of anemia. The normal function of certain heme-containing enzymes is also affected by iron deficiency.

Venn et al. ('47) estimated that the body of the pig at birth contains approximately 50 mg of iron of which the pig must retain about 7 mg per day to grow at a normal rate without becoming anemic. It is well recognized that the efficiency of utilization of orally administered iron is dependent upon many exogenous factors, as well as the state of iron depletion, and that much more iron must be supplied *per os* than is necessary for normal metabolic functions. The following experiment was undertaken to determine the oral iron requirement of the baby pig under carefully controlled conditions.

MATERIALS AND METHODS

Three trials were conducted using 45 pigs of both sexes, including Durocs, Chester Whites and cross-breeds. The pigs were removed from the sow when one-week old and placed on a synthetic milk diet, unsupplemented with iron, for 4 days to one week. At the end of this time, they were assigned at random by litter and weight to the various treatments.

The basal synthetic milk diet contained 25 parts of iron per million parts of solids. During the first trial, the pigs were individually fed this milk supplemented with zero, 10 or 100 ppm of ferrous iron ($FeSO_4 \cdot 7H_2O$).[3] During the second trial, milk supplemented with zero, 50, 100 or 200 ppm was provided. The third trial was a replicate of the second. Experimental diets were fed for 6 weeks in all trials.

De-ionized, distilled steam condensate was used in formulating the synthetic milk. The solids, composing 20% of the milk, consisted of the following in per cent: cerelose, 52.5; "vitamin-free" casein,[4] 30; lard, 10; minerals, 6; $NaHCO_3$, 1.5; and vitamins. With the exception of iron, minerals and vitamins were supplied in the same quantities used by Miller et al. ('54).

The pigs were housed in galvanized, wire-bottom cages spray-painted with aluminum paint.

In a search for the most sensitive measure of dietary adequacy, the following observations were made at either weekly or biweekly intervals: weight gain, hemoglobin concentration, hematocrit, erythrocyte count, reticulocyte count, leucocyte count, total serum protein, serum protein electrophoretic pattern, serum-iron concentration, unbound iron-binding capacity of serum, total iron-binding capacity of serum and per cent saturation of transferrin with iron.

Blood was obtained from the anterior vena cava using equipment washed free of iron with repeated applications of 25% HCl and double-distilled, de-ionized steam condensate.

Hemoglobin was determined using the cyanmethemoglobin method of Crosby et al. ('54).

Hematocrit was determined according to the micromethod described by McGov-

Received for publication June 22, 1959.

[1] Published with the approval of the Michigan Agricultural Experiment Station as Journal Article no. 2455.

[2] The authors wish to acknowledge the assistance of R. E. Samson and E. D. Purkhiser in feed formulation and animal care, and of R. M. Diener in performing the bone-marrow biopsies.

[3] All iron supplements are based on the solids content of the diet.

[4] Labco.

ern et al. ('55). Blood samples were centrifuged for 5 minutes at 10,000 rpm in an International "Hemacrit" centrifuge.

Erythrocytes were counted in duplicate from a single filling of a "zero error" Hellige pipette using 0.85% (W/V) NaCl as the diluting fluid. Cells were counted in a hemocytometer with Neubauer ruling using a National Bureau of Standards certified cover glass. Acceptable duplicate counts differed no more than 8% of the lower count.

Reticulocytes were enumerated per 1000 erythrocytes in blood smears vitally stained with brilliant cresyl blue and counterstained with Wright's stain.

Total serum protein was determined using the biuret procedure accepted by the American Association of Clinical Chemists (Reiner, '53). Pooled porcine serum was standardized by macroKjeldahl methods.

Electrophoretic separation of serum proteins was accomplished on a Spinco Model R paper electrophoresis system.[5]

Methods for determination of serum iron, unbound-iron binding capacity, total-iron binding capacity and transferrin-iron saturation were adapted from Schade et al. ('54). Sulfonated 4,7-diphenyl-1,10-phenanthroline[6] was used as the chromagenic reagent. All spectrophotometric measurements were made with a Beckman DU using 1-cm cuvettes.

During the latter two weeks of trials 1 and 3, 21 pigs were placed in metabolism cages for a 4- or 5-day quantitative collection of excreta. Feed intake was kept constant during the collection and for the two days immediately preceding. Excreta were collected in iron-free glassware. Iron assay methods were adapted primarily from Bandemer and Schaible ('44). Excreta were wet-ashed with concentrated H_2SO_4 and HNO_3. Ammonium persulfate was used to destroy nitrosyl sulfuric acid which formed in the presence of high amounts of calcium. The chromogenic reagent used was α, α-dipyridyl.

Bone marrow biopsies were performed on two pigs per treatment at the end of trial 3. Marrow was obtained from the sternum and tibia for differential cell counts of Wright's-stained smears. Smears were also stained with potassium ferrocyanide according to the method of Rath and Finch ('48) to detect the presence of hemosiderin.

Twenty-one pigs from trials 1 and 3 were exsanguinated after anesthesia with CO_2 or sodium pentobarbital and were examined carefully, grossly and histologically. Liver, spleen, kidney and gastrocnemius muscle were analyzed for iron content, using the same procedures as for excreta.

Statistical analyses of the data were performed according to the methods of Snedecor ('46) using the *t* test as the fiducial measure.

RESULTS AND DISCUSSION

Weight gains and feed consumption. Data relative to weight gains and feed consumption are presented in table 1. Pigs receiving 50, 100 or 200 ppm of supplementary iron gained significantly faster than those in the unsupplemented group. Daily feed consumption was significantly higher for pigs receiving 100 or 200 ppm of supplementary iron compared with the unsupplemented controls. Also, the kilograms of solids per kilogram of gain were significantly less than those found for the controls in all supplemented groups.

Hematology. The effect of added iron on certain erythrocyte measurements is illustrated in table 2. Measurements of hemoglobin and hematocrit were early, sensitive indicators of the state of iron repletion. Erythrocyte counts and mean corpuscular hemoglobin concentrations were less useful measurements, but these, plus the marked change in mean corpuscular volume, also supported the conclusion that 100 ppm of supplemental iron was an adequate level.

Reticulocyte concentration was determined only in trial 3 at two-week intervals. The first determination probably was not made soon enough after initiation of treatment to reveal significant differences. Total leucocytes were counted only in trial 1. These values noted in all pigs were within the normal range and treatment did not effect significant differences.

[5] Spinco Technical Bulletin 6026A.

[6] Bathophenanthroline, G. Frederick Smith Chemical Company, Columbus, Ohio.

Serum proteins. Table 3 illustrates the effect of added iron upon serum proteins. The most significant alteration observed was an increase of the percentage of beta globulin in serum obtained from pigs receiving inadequate iron. This change correlated well with the increase in serum total-iron-binding capacity. Since transferrin is a specific beta-1 globulin, such an effect was not unexpected.

TABLE 1

Weight gain and feed consumption of young pigs fed different amounts of iron
(Trials 1, 2 and 3)

Observations during 6 weeks on experiment	Added iron, ppm				
	0	10	50	100	200
Number of pigs	12	4	8	14	7
Initial weight, *kg*	2.5	2.6	2.5	2.4	2.4
Daily gain, *kg*	0.18	0.20	0.21[1]	0.22[1]	0.24[2]
Daily solids consumed, *kg*	0.22	0.24	0.24	0.25[1]	0.28[2]
Solids per kilogram gain, *kg*	1.29	1.19[1]	1.21[1]	1.17[2]	1.21[1]

[1] Significantly different from unsupplemented group ($P < 0.05$).
[2] Significantly different from unsupplemented group ($P < 0.01$).

TABLE 2

Effect of orally-administered iron on erythrocytes of young pigs
(Trials 1, 2 and 3)

Weeks on treatment	Added iron, ppm				
	0	10	50	100	200
			Hemoglobin, gm/100 ml blood		
0	7.3	7.0	7.0	7.4	7.4
2	6.0	6.6	7.0	8.6[1]	8.6[1]
4	4.6	6.3[2]	8.5[3]	10.3[1]	11.7[1]
6	3.4	6.2[4]	7.9[4,5]	11.6[1]	11.7[1]
			Hematocrit %		
0	24.3	21.9	24.7	24.2	25.9[2]
2	20.2	20.1	25.9[3]	29.5[3,6]	30.1[3,6]
4	17.3	22.1[2]	28.2[3]	32.5[3]	37.4[1]
6	13.3	20.7[4]	27.6[3]	36.8[1]	37.8[1]
			Erythrocytes, millions/cubic millimeter		
0	4.87	4.56	5.43	4.92	5.52
2	5.24	5.34	6.75[3]	6.44[3]	7.04[3]
4	4.83	5.63	6.91[4,5]	6.66[4,5]	7.33[3]
6	3.67	5.54[4]	6.56[4]	6.95[4,5]	6.74[4,5]
			Mean corpuscular hemoglobin concentration, %		
0	30.2	31.8	28.4	30.3	28.6
2	30.1[2]	33.1[4,5]	27.0	29.1	28.5
4	26.0	28.7[2]	29.1[4]	30.9[4]	31.3[4]
6	26.0	30.1[4]	28.5[2]	31.4[4]	31.1[4]
			Mean corpuscular volume, cubic microns		
0	50.6	48.3	45.5	49.2	47.5
2	38.8	37.8	38.6	45.9[1]	42.9[6]
4	37.3	39.7	40.7	50.4[1]	51.0[1]
6	36.5	38.6	42.8[2]	53.4[1]	56.5[1]

[1] Significantly greater than least three values ($P < 0.01$).
[2] Significantly greater than least value ($P < 0.05$).
[3] Significantly greater than least two values ($P < 0.01$).
[4] Significantly greater than least value ($P < 0.01$).
[5] Significantly greater than least two values ($P < 0.05$).
[6] Significantly greater than least three values ($P < 0.05$).

TABLE 3

Effect of orally-administered iron on serum proteins of young pigs

(Trial 3)

Weeks on treatment	Added iron, ppm			
	0	50	100	200
	Number of pigs			
	5	5	6	4
	Total serum protein, gm/100 ml serum			
0	6.1	6.4	6.4	6.7
2	5.2	5.2	5.0	5.3
4	5.2[1]	4.3	4.8	4.8
6	5.2	5.0	4.9	5.1
	Serum albumin, % of total serum protein			
0	40.4	42.5	45.6	44.3
2	46.3	45.0	47.6	50.7
4	49.6	49.0	52.2	54.8[2]
6	51.9	53.4	57.1[3]	58.1[3]
	Serum alpha globulin, % of total serum protein			
0	21.5	22.1	22.4	21.1
2	23.3	24.3	22.5	22.2
4	22.4	24.1	22.0	20.6
6	23.8	22.4	21.0	20.9
	Serum beta globulin, % of total serum protein			
0	15.1	15.5	15.3	16.2
2	17.7	16.9	17.4	16.6
4	21.1[4]	17.7	16.4	15.6
6	19.7[1,5]	16.8[3]	14.4	15.3
	Serum gamma globulin, % of total serum protein			
0	23.0	20.0	16.7	18.4
2	12.6	13.1	11.8	10.5
4	7.0	9.1	9.4[3]	9.0
6	4.8	7.4	7.3	5.5

[1] Significantly greater than least three values ($P < 0.05$).
[2] Significantly greater than least two values ($P < 0.05$).
[3] Significantly greater than least value ($P < 0.05$).
[4] Significantly greater than least three values ($P < 0.01$).
[5] Significantly greater than least two values ($P < 0.01$).

Serum iron. Supplementary iron produced significant effects upon the values presented in table 4. Pigs receiving 100 or 200 ppm of supplementary iron exhibited highest serum iron concentration and transferrin saturation and lowest serum unbound and total iron-binding capacity.

Iron metabolism. Efficiency of iron utilization was increased in trial 1 when no supplemental iron was administered. Urinary iron excretion was not significantly affected by the treatment, but the percentage of ingested iron recovered in the feces was 17.8, 28.6 and 50.1, respectively, for supplements of zero, 10 and 100 ppm of iron. The difference between the 100 ppm treatment and other levels was significant ($P < 0.05$). Results in trial 3 were inconclusive owing to a considerable amount of individual variation in fecal iron excretion.

Bone marrow. No significant differences in bone marrow cell distribution were observed, nor evidence of stainable iron deposits as hemosiderin.

Tissue iron. As illustrated in table 5, supplementary iron increased the iron concentration of the muscle to levels higher than that observed in the unsupplemented pigs. Animals receiving 100 or 200 ppm exhibited the highest muscular iron concentration. Both the liver iron concentration and the total liver iron were significantly higher in pigs supplemented with

TABLE 4

Effect of orally-administered iron on serum iron of young pigs

(Trial 3)

Weeks on treatment	Added iron, ppm			
	0	50	100	200
	Number of pigs			
	5	5	6	4
	Serum iron, µg/100 ml serum			
0	22	21	20	28
2	26	69	110[1]	257[2]
4	23	96	326[3]	520[2]
6	53	225[1]	445[3]	449[3]
	Serum unbound iron-binding capacity, µg/100 ml serum			
0	616	653	692	740
2	589[4]	495[4]	448[1]	252
4	770[2]	489[3]	214[1]	47
6	681[2]	314[5]	136	103
	Serum total iron-binding capacity, µg/100 ml serum			
0	656	673	726	720
2	615	564	558	508
4	793[2]	585	540	566
6	734[2]	539	569	552
	Saturation of transferrin with iron, %			
0	3.2	3.1	2.9	4.0
2	4.2	12.5	20.2[1]	53.6[3]
4	2.9	15.9	59.9[3]	90.5[3]
6	7.6	42.7[4]	82.0[3]	81.2[3]

[1] Significantly greater than least value ($P < 0.05$).
[2] Significantly greater than least three values ($P < 0.01$).
[3] Significantly greater than least two values ($P < 0.01$).
[4] Significantly greater than least value ($P < 0.01$).
[5] Significantly greater than least two values ($P < 0.05$).

TABLE 5

Effect of orally-administered iron on tissue iron of young pigs

(Trial 3)

	Added iron, ppm			
	0	50	100	200
No. of pigs	2	2	2	2
Muscle				
Fe/gm, *µg*	6.6	10.5[1]	12.2[1,2]	12.5[1,2]
Liver				
Organ weight, *gm*	309	414	414	370
Fe/gm, *µg*	19.2	22.6	31.6	54.2[2]
Total Fe, *mg*	6.0	9.3	13.1	20.1[3]
Kidney				
Organ weight, *gm*	82	95	84	101
Fe/gm, *µg*	22.4	29.9	38.4	36.1
Total Fe, *mg*	1.8	2.9	3.2	3.6
Spleen				
Organ weight, *gm*	30	42	21	24
Fe/gm, *µg*	122.2	80.1	72.3	93.5
Total Fe, *mg*	3.6	3.4	1.5	2.4

[1] Significantly greater than least value ($P < 0.01$).
[2] Significantly greater than least two values ($P < 0.05$).
[3] Significantly greater than least value ($P < 0.05$).

200 ppm of iron than in the unsupplemented control animals. The possibility that these differences might be caused by residual blood, which varied markedly in hemoglobin concentration with respect to treatment, rather than true tissue iron has not been resolved.

Comparison with National Research Council recommendations. The National Research Council, in their 1958 report, indicated that 10 to 15 mg of iron daily for the first 6 weeks after birth would be expected to maintain adequate levels of hemoglobin in suckling pigs. Our data indicated that the blood picture of baby pigs which were initially anemic (approximately 7 gm % of hemoglobin) did not deteriorate further when only 75 ppm of ferrous iron were present in the dietary solids, nor did their blood picture improve. Based on a daily intake of 0.24 kg of solids, the daily iron intake would have been 18 mg. Using the criteria previously described and under the specified conditions, where the subjects are initially anemic, it appears that 125 ppm of oral iron (100 ppm as $FeSO_4 \cdot 7H_2O$ plus 25 ppm in the basal diet) is more nearly adequate for the baby pig.

SUMMARY

Forty-five baby pigs were fed a synthetic milk diet (25 parts of iron per million parts of solids) supplemented with zero, 10, 50, 100 or 200 parts of ferrous iron ($FeSO_4 \cdot 7H_2O$) per million parts of solids. The following observations were made at weekly or biweekly intervals: weight gain, hemoglobin concentration, hematocrit, erythrocyte count, reticulocyte count, leucocyte count, total serum protein, serum protein electrophoretic pattern, serum iron concentration, unbound iron-binding capacity of serum, total iron-binding capacity of serum and percentage saturation of transferrin with iron. Iron balance studies, bone marrow biopsies and tissue analyses were also performed. Under the specified conditions, 125 ppm of oral iron appear adequate for the baby pig.

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The Effect of Sulfate on Molybdenum Toxicity in the Chick[1]

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The toxicity of sodium molybdate for the chick has been described by Kratzer ('52), Motzok, Arthur and Branion ('57) and Arthur, Motzok and Branion ('58). These workers reported that growth was depressed when high levels of molybdenum were added to practical diets. The lowest molybdenum level at which depression occurred varied from 100 ppm (Kratzer, '52) to 500 ppm (Motzok et al., '57). Although cattle (Ferguson et al., '38), sheep (Dick, '52) and rabbits (Arrington and Davis, '53) all suffer from anemia during molybdenum toxicosis, no changes in blood constituents were observed in chicks fed up to 4000 ppm of molybdenum (Arthur, Motzok and Branion, '58).

Copper has been employed to alleviate molybdenum toxicity in several species. Supplementation of diets with copper sulfate reduced molybdenum-induced growth depression in chicks (Kratzer, '52; Arthur, Motzok and Branion, '58), rats (Gray and Daniel, '54) and ruminants (Ferguson et al., '43). Dick ('53a), however, pointed out that inorganic sulfate affected molybdenum metabolism in sheep, and that the anion should be considered in evaluating the effects of copper sulfate. The possibility that organic sulfur might act in a similar manner was suggested by the observation of Gray and Daniel ('54) that methionine was also effective in counteracting molybdenosis in rats.

The experiments to be reported were designed to study the toxic symptoms produced when molybdenum was added to a purified chick diet, and to examine the relationship between molybdenum and inorganic sulfate.

PROCEDURE

Day-old straight-run New Hampshire chicks were wing-banded and distributed at random in groups of 18 to 20 in electrically-heated battery brooders. Water and experimental diets were provided ad libitum. The purified basal diet used in all experiments was that reported by Reid et al. ('56). Supplements were added at the expense of glucose monohydrate.[2]

Experiment 1 consisted of three tests in which levels of from 100 to 8000 ppm of molybdenum were fed to chicks. Mortality and individual weight of all chicks was recorded at 4 weeks. At the end of this period, 10 birds were selected at random from each group in tests 1b and 1c. Blood hemoglobin level of each bird was determined by the alkaline hematin method, and packed cell volume (in triplicate) by centrifuging in heparinized capillary tubes. Total cell count was also obtained in test 1c.

Experiment 2 was designed to examine the relationship between high levels of molybdenum and sulfate (as sodium sulfate) in two tests. Weight and mortality were recorded at 4 weeks. Hemoglobin

Received for publication July 31, 1959.

[1] This work was supported in part by a grant from the Robert A. Welch Foundation, Houston, Texas.

[2] The following products were supplied through the courtesy of the indicated companies: inositol by Corn Products Refining Co., Argo, Illinois; folic acid and Aureomycin by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.; biotin by Hoffman-La Roche, Inc., Nutley, N. J.; soybean oil by Buckeye Cellulose Corp., Cincinnati; methionine and glycine by Dow Chemical Co., Freeport, Texas; stabilized vitamin A by Stabilized Vitamins, Inc., Garfield, N. J.; vitamin D_3 by Bowman Feed Products, Co., Holland, Mich.; α-tocopheryl acetate, riboflavin, thiamin·HCl, D-calcium pantothenate, p-aminobenzoic acid, pyridoxine·HCl, niacin, choline chloride, and vitamin B_{12} by Merck, Sharp and Dohme, Rahway, N. J., and menadione sodium bisulfite by Heterochemical Corp., Valley Stream, N. Y.

and packed cell volume of blood from 10 birds per group were measured in test 2b.

To avoid confusion between cationic and anionic effects, experiment 3 compared the effects of sodium and ammonium salts of both molybdenum and sulfate, in all possible combinations, on growth and mortality. Hemoglobin and packed cell volume were measured in the 4 groups receiving either the basal diet or basal diet plus sodium salts.

In experiment 4, graded levels of sulfate, from 1000 to 8000 ppm, were added to a diet containing 2000 ppm of molybdenum. The concentration of molybdenum in tibia shafts from 10 birds per group was measured by the method of Marmoy ('39). Two additional groups were fed diets containing 0.62% of added methionine, in the presence and absence of molybdenum. This level of methionine provided sulfur equivalent to that contained in 4000 ppm of sulfate. Weight and mortality were recorded at 4 weeks.

RESULTS

The lowest level of molybdenum which produced a growth depression was 500 ppm (table 1). As the molybdenum level was increased this depression became more severe, until at the highest level (8000 ppm) 4-week weight was one-sixth that of the basal group. No appreciable mortality was caused by feeding at levels up to 4000 ppm, but 6000 and 8000 ppm of molybdenum proved lethal: mortality was 33 and 61%, respectively.

Addition of 500 to 2000 ppm of molybdenum caused a small but consistent elevation of both hemoglobin concentration and packed cell volume. At the 2000 ppm level the red cell count was also elevated. Higher concentrations of molybdenum produced anemia: cell volume and cell count of birds receiving 4000 ppm were below the basal group values, and hemoglobin concentration was depressed at 6000 ppm molybdenum. The slight increase in all three measurements at 8000 ppm was not surprising, since the 7 birds which survived the experimental period would not constitute a representative sample.

Addition of sulfate, as sodium sulfate, to diets containing toxic levels of molybdenum greatly reduced growth depression (table 2). When a diet containing 4000 ppm of molybdenum was supplemented with 8000 ppm of sulfate, anemia was almost eliminated. The hemoglobin level was elevated to a value slightly higher than

TABLE 1

Effect of molybdenum on growth and blood constituents in chicks

Test	Molybdenum added[1]	4-week weight	Mortality	Hemoglobin[2]	Packed cell volume[2]	Red blood cells[2]
		gm	%	*gm/100 ml*	%	$10^6/mm^3$
1a	None	311	0			
	100	323	5			
	200	305	0			
	300	302	0			
	400	316	0			
	500	291	10			
1b	None	352	0	7.72 ± 0.433	31.2 ± 1.61	
	500	313	5	8.04 ± 0.326	32.3 ± 1.12	
	750	274	0	7.64 ± 0.404	33.1 ± 2.79	
	1000	260	0	7.84 ± 0.486	33.0 ± 1.52	
	1250	263	0	8.26 ± 0.435	33.2 ± 1.80	
	1500	230	0	8.22 ± 0.488	32.6 ± 1.89	
1c	None	301	0	6.49 ± 0.649	29.9 ± 1.49	2.31 ± 0.140
	2000	194	6	7.58 ± 0.487	32.5 ± 2.02	2.54 ± 0.166
	4000	103	6	6.78 ± 0.454	26.9 ± 2.25	2.24 ± 0.083
	6000	64	33	5.45 ± 0.516	25.8 ± 2.22	1.83 ± 0.134
	8000	47	61	5.82 ± 0.315	26.9 ± 0.98	1.94 ± 0.103

[1] As $Na_2MoO_4 \cdot 2H_2O$.

[2] Mean ±95% confidence interval.

that of the basal group, while packed cell volume and cell count were only slightly below the basal values. Weights and blood measurements for the first 4 groups of experiment 3 are included in table 2 for comparison. As in the case of the lower levels of molybdenum, addition of sulfate in a ratio of 2:1 reduced the growth depression and anemia produced by feeding 6000 ppm of molybdenum.

TABLE 2

Effect of sulfate on molybdenum toxicity in chicks

Test	Treatment	4-Week weight	Mortality	Hemoglobin[3]	Packed cell volume[3]	Red blood cells[3]
		gm	%	*gm/100 ml*	%	$10^6/mm^3$
2a	Basal	311	0			
	2000 ppm Molybdenum[1]	189	0			
	4000 ppm Sulfate[2]	326	5			
	2000 ppm Molybdenum + 4000 ppm sulfate	258	5			
2b	Basal	345	5	7.07 ± 0.391	31.2 ± 1.76	2.75 ± 0.106
	4000 ppm Molybdenum	86	0	4.86 ± 0.776	25.8 ± 2.72	2.14 ± 0.131
	8000 Sulfate	328	5	7.31 ± 0.528	31.8 ± 2.71	2.79 ± 0.243
	4000 Molybdenum + 8000 ppm sulfate	209	0	7.49 ± 0.513	29.8 ± 1.49	2.38 ± 0.127
3	Basal	346	0		31.6 ± 2.41	
	6000 ppm Molybdenum	68	24		28.4 ± 2.18	
	12,000 ppm Sulfate	329	0		33.6 ± 1.88	
	6000 ppm Molybdenum + 12,000 ppm sulfate	162	4		32.0 ± 1.44	

[1] As $Na_2MoO_4 \cdot 2H_2O$.
[2] As Na_2SO_4.
[3] Mean ±95% Confidence limits.

TABLE 3

Effect of molybdenum and sulfate, as sodium and ammonium salts, on growth and mortality in chicks

Treatment	4-Week weight	Mortality
	gm	%
Basal	346	0
6000 ppm Molybdenum S[1]	68	24
12000 ppm Sulfate S[2]	329	0
6000 ppm Molybdenum S + 12000 ppm sulfate S	162	4
6000 ppm Molybdenum A[3]	66	48
12000 ppm Sulfate A[4]	299	0
6000 ppm Molybdenum A + 12000 ppm sulfate A	102	17
6000 ppm Molybdenum A + 12000 ppm sulfate S	147	22
6000 Molybdenum S + 12000 ppm sulfate A	96	8

[1] Molybdenum S: as $Na_2MoO_4 \cdot 2H_2O$.
[2] Sulfate S: as Na_2SO_4.
[3] Molybdenum A: as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.
[4] Sulfate A: as $(NH_4)_2SO_4$.

The general effect of ammonium sulfate on molybdenum-induced growth depression was similar to that of sodium sulfate (table 3). The ammonium ion was somewhat toxic at the level (4500 ppm) present in the sulfate salt. Ammonium sulfate appeared to be equally as effective as sodium sulfate in preventing excessive mortality. Groups fed ammonium molybdate consistently suffered a higher mortality than the corresponding ones fed sodium molybdate. This additional mortality was not affected by the addition of sulfate to the diet; the mortality in groups fed ammonium molybdate plus some form of sulfate was approximately equal to the difference in mortality between groups fed the sodium and ammonium molybdate salts.

Successive increases in the amount of sulfate added to a high-molybdenum diet resulted in progressively improved growth (table 4). At the highest sulfate level used, slightly more than one-half of the growth depression was overcome. Methionine was completely ineffective and produced a growth depression when added to either the basal or the high-molybdenum diet (table 4).

Although sulfate reduced growth depression, it did not reduce the storage of molybdenum in the tibiae. Groups fed 2000 ppm of molybdenum had approximately 100 times the concentration of molybdenum in bone ash compared with those fed low-molybdenum diets, regardless of the level of sulfate in the diet. Adding either 4000 or 8000 ppm of sulfate to a low-molybdenum diet had little effect, either on growth or on tibia molybdenum storage.

None of the birds fed high-molybdenum diets in these experiments showed any evidence of diarrhea. This agrees with the observations of Kratzer ('52) concerning lower molybdenum levels. No gross physical changes, other than retarded growth, were observed in these birds. Although some weakness was noted during the second and third week, in the groups fed lethal levels of the mineral, all survivors appeared quite vigorous at the end of the experiment. Skeletal and muscular development appeared to be equally retarded; the birds were not emaciated. Post-mortem examination revealed no gross changes other than generally pale viscera in the birds.

DISCUSSION OF RESULTS

The minimal toxic dose of molybdenum in the present series of experiments was 500 ppm. This observation agrees with that of Motzok, Arthur and Branion ('57), but is higher than toxic levels reported by Kratzer ('52) and by Arthur, Motzok and Branion ('58). These variations are very probably due to differences in the diets used, whereas the previous observations were made with practical diets. Since sulfate reduced the toxicity of molybdenum, it may be significant that the purified diet used in the present experiments contained 2667 ppm of *added* sulfate as the magnesium, manganese and copper salts.

TABLE 4

Effect of methionine and of graded levels of sulfate on molybdenum toxicity in chicks

Treatment	4-Week weight	Tibia molybdenum
	gm	*µg/gm. bone ash*
Basal	328	2.36
2000 ppm Molybdenum[1]	222	192.04
2000 ppm Molybdenum + 1000 ppm sulfate[2]	232	199.40
2000 ppm Molybdenum + 2000 ppm sulfate	250	235.42
2000 ppm Molybdenum + 4000 ppm sulfate	257	194.10
2000 ppm Molybdenum + 8000 ppm sulfate	278	217.11
4000 ppm Sulfate	348	2.52
8000 ppm Sulfate	325	1.11
0.62% Methionine	297	
2000 ppm Molybdenum + 0.62% methionine	210	

[1] As $Na_2MoO_4 \cdot 2H_2O$.

[2] As Na_2SO_4.

Whereas it is not possible to estimate the sulfate content of the practical diets, it is probable that manganese sulfate was the only major *added* source of this anion; even at a level of one-half pound per ton, manganese sulfate would contribute only about 150 ppm of sulfate.

The investigators cited previously did not find any evidence of anemia in chicks suffering from molybdenum toxicosis. The highest molybdenum level fed by Kratzer ('52) was 400 ppm, which would not be expected to produce anemia according to the results reported here. Arthur, Motzok and Branion ('58), however, fed as much as 4000 ppm of molybdenum without causing a reduction in hemoglobin or packed cell volume. Since 4000 ppm appeared to be the lowest anemia-producing level in the purified diet, and since hemoglobin level was the variable most resistant to reduction, dietary differences might possibly account for the contrasting observations.

The fact that sodium sulfate and ammonium sulfate had similar effects on molybdenum-induced growth depression and mortality, strongly implicates sulfate as the responsible ion. The ammonium ion was toxic at 4500 ppm, the level provided by ammonium sulfate, but was not lethal. The increased mortality observed when ammonium molybdate replaced the sodium salt could not be due to ammonium ion, since only 1169 ppm was introduced as the molybdate. The form of the anion might be the critical differences between sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) and ammonium molybdate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$).

The data obtained in experiment 4 suggest that a sulfate:molybdenum ratio greater than 4:1 might be expected to reduce growth depression further. This experiment failed, however, to indicate the mechanism of sulfate action in chicks. There was no evidence of reduced molybdenum storage in the presence of high levels of sulfate. This observation was contrary to the report by Dick ('53a) that increased dietary sulfate reduced the storage of molybdenum in all tissues of sheep. Dick found that molybdenum concentration in the skeleton was reduced by 80 to 90% in 35 days when moderate levels of sulfate were fed. In this experiment however, the ratios of sulfate to molybdenum intake were as high as 21,000 to 1, and this factor might be more important than species differences in accounting for the differing results.

The failure of methionine to alleviate molybdenum toxicity was contrary to the effect reported in rats. Gray and Daniel ('54) found that 0.6% or 1.2% methionine greatly improved the growth of rats receiving 800 ppm of molybdenum. The higher level of methionine was reported to be toxic for the rat. Similarly 0.62% methionine was toxic for the chick in the present experiments, but there was no evidence of a molybdenum-methionine interaction such as that observed by Gray and Daniel. Thus it is not possible to attribute the effect of sulfate to a sparing action of methionine.

The effectiveness of sulfate in reducing molybdenum toxicity suggests that the reported effects of copper, as copper sulfate, be re-evaluated considering both anion and cation. At present no evidence exists to suggest a possible mechanism of the sulfate effect. Since high levels of molybdenum produced anemia, and high levels of sulfate countered this effect, possibly the metabolism of either copper or iron is affected. Dick ('53b) found that the dietary level of both molybdenum and sulfate was critical in determining the status of copper stores in sheep. Further studies have been undertaken in an attempt to clarify possible relationships in the chick.

SUMMARY

Molybdenum, as sodium molybdate, was toxic for chicks when added to a purified diet at levels of 500 ppm or higher. A slight but consistent elevation in hemoglobin concentration and packed cell volume was observed at molybdenum levels between 500 and 2000 ppm. Anemia was observed in birds receiving 4000 ppm or more of molybdenum. At 6000 to 8000 ppm, molybdenum caused high mortality. Ammonium molybdate produced higher mortality than the sodium salt.

Sulfate, added to the diets at twice the concentration of molybdenum, alleviated

all physical symptoms of molybdenosis. Sodium and ammonium sulfate had similar effects, although the ammonium salt exhibited toxic properties. The sulfate salts practically eliminated the mortality produced by sodium molybdate, but failed to prevent the additional mortality characteristic of ammonium molybdate. Addition of graded levels of sulfate to a high-molybdenum diet failed to prevent greatly increased storage of molybdenum in the tibiae of chicks, although growth improved as sulfate concentration was increased.

Methionine failed to affect the growth depression caused by a high level of molybdenum.

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Toxicity of Air-Oxidized Soybean Oil[1,2]

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Although it has long been recognized that overheated or oxidized fat causes toxic manifestations in the rat, neither the exact nature of materials causing these symptoms nor the specific mechanisms by which they are caused have been understood with any certainty. Work prior to 1940 established that highly oxidized fat accelerates the destruction of a large variety of essential nutrients (Burr and Barnes, '43). Subsequent work, however, has indicated that certain oxidized oils contain materials which are toxic to the rat even though apparently adequate steps are taken to protect the easily-oxidizable foodstuffs in the diet.

The identity of the toxic materials in oxidized or overheated fat has been investigated by two routes, differing in the manner by which the oxidized fat was obtained. Several groups of investigators, including Crampton et al. ('51a, '51b, '51c, '53, '56), Common et al. ('57), Granados et al. ('49), and Raju and Rajagopalan ('55) have changed the chemical nature of oils primarily by means of heat treatment, often with complete exclusion of air. Other groups of investigators (Matsuo, '54; Kaneda et al., '55; Kaunitz et al., '55; Andrews et al., '56) oxidized various oils by aeration with little or no elevation in temperature during the oxidation process. Grossly, the results obtained are similar when either type of oxidized oil is fed, namely, rough fur and unkempt appearance and decreased weight gain often followed by death. When, however, those groups feeding heat-treated oil measured the peroxide content of their product, the peroxide concentration in the toxic oil was found to be very low compared with values obtained with aerated oil. On the other hand, in those cases in which oils were oxidized by aeration and some attempt was made to measure the toxicity due to polymers, the polymer content appeared completely innocuous, the toxicity apparently correlating best with peroxide concentration.

In the experiments to be reported in this paper, an attempt was made to reconcile these apparently contradictory results and, further, to identify the toxic mechanism or mechanisms which so frequently lead to death in the rat. There are apparently few or no definitive data on the latter point although it would seem to be of considerable importance in many areas of the world in which cooking customs involve intermittent high-temperature and open air heating of highly unsaturated oils (Raju and Rajagopalan, '55). Consequently, in a preliminary experiment, various levels of peroxidized fat were fed to determine the levels of toxicity and toxic symptoms. Second, the peroxidized oil was fractionated in such a way as to separate peroxide and polymeric material and the fractions were fed. Third, the absorption of peroxidized fat was studied, and fourth, an attempt was made to ascertain the site of the damage.

METHODS AND MATERIALS

Growth experiments. Unless otherwise noted, weanling rats[4] were fed diets containing air-oxidized soybean oil. The animals were individually housed in sus-

Received for publication May 2, 1959.

[1] This paper reports research undertaken in cooperation with the Office of the Surgeon General under contract DA-49-007-MD-579.

[2] A preliminary report of a portion of this work was presented at the annual meeting of the Federation of American Societies for Experimental Biology, April 19, 1956.

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pended cages and, where pertinent, individual diet consumption records were kept. Each rat was weighed twice weekly.

Two diets were utilized in the growth experiments. The percentage composition of diet A was as follows: casein, 25; sucrose, 46; salt mixture, 4; brewers' yeast, 5; fat, 20. The composition of diet B is presented in table 1, as well as the composition of the vitamin and salt mixtures.

Semiweekly administration to each rat of 175 U.S.P. units of vitamin A, 35 units of vitamin D and 1.75 mg of α-tocopherol acetate dissolved in 0.05 ml of sesame oil was utilized to satisfy the fat-soluble vitamin requirement. Initially, this supplement was administered by intramuscular injection in order to avoid the possibility of oxidation by peroxide in the gut. Subsequent experimentation, however, established that similar growth could be obtained in rats given this supplement orally at noon on a semiweekly basis (most of the diet was consumed during the night), and this method of supplementation was used in later experiments.

Preparation of oxidized soybean oil. The raw oil was oxidized by aeration in a water bath at 60°C for approximately one week with the addition of 2 mg/kg each of $CuCl_2$ and $FeCl_2$. Toward the end of the oxidation period the peroxide number (PN), milliequivalents of peroxide oxygen per kilogram of oil, was determined at intervals according to the method of Polister and Mead ('53) so that oxidation might be halted at maximum peroxide concentration inasmuch as the PN decreases rapidly after the maximum is reached. The oxidized oil was then stored at −20°C under nitrogen.

Fractionation of oxidized soybean oil. Two kilograms of raw oil were oxidized in the usual manner and one-half of the oxidized oil was stored at −20°C (fraction C). The remaining oil was dissolved in 1.7 l of petroleum ether (b.p. 30 to 60°) saturated with methanol and was then extracted with 4 l of methanol saturated with petroleum ether. The oil contained in the petroleum ether layer was separated on a silicic acid column into two fractions. This fractionation was accomplished by adding 25-gm quantities of oil in petroleum ether to 8 by 14-cm silicic acid columns and eluting with 500-ml portions of 5% ethyl ether in petroleum ether. Each fraction was collected separately and evaporated to dryness under nitrogen in a warm-water bath. All fractions weighing more than 1 gm were tested for peroxide, as previously described, and those fractions having a peroxide number of less than 20 were pooled. The resulting colorless oil (fraction A) had a peroxide value of 13.2 and contained little or no polymeric material as shown by complete distillation of a methylated sample. After removal of the low-peroxide material from the columns, the remaining oil was eluted with three- to 4-column volumes of methanol. The methanol was evaporated under reduced pressure at a temperature under 40°C. The last traces of solvent were removed under high vacuum at room temperature. The combined sample from all

TABLE 1
Composition of diet B

Dietary component main mixture		Salt mixture		Vitamin mixture	
		Salt	Amount	Vitamin	Amount
	gm/100 gm		*gm*		*mg/gm*
Oxidized oil	15.0	$CaCO_3$	300.0	Choline	120,000
Casein	18.0	$CaHPO_4$	75.0	Thiamine	200
Sucrose	60.8	K_2HPO_4	322.5	Riboflavin	200
Glycerol	1.0	NaCl	167.4	Niacin	400
l-Cystine	0.2	$MgSO_4$	49.8	Calcium pantothenate	800
Salt mixture	4.0	$MnSO_4 \cdot 2H_2O$	0.5	Folic acid	8
Vitamin mixture	1.0	Zn acetate	0.402	Biotin	4
		Fe citrate	27.4	Pyridoxine	100
		KI	0.8	Vitamin B_{12}	0.8
		$CuSO_4 \cdot 5H_2O$	0.3	*i*-Inositol	4.0
				Sucrose	273,500

of the columns was an amber, moderately viscous oil with a peroxide number of 927 (fraction B). The original methanol fraction was extracted with petroleum ether at 0°C for 6 hours in a liquid-liquid extractor. The sample, in methanol, was stored under nitrogen at −20°C until shortly before use. The methanol was then removed under reduced pressure in an ice bath with a slow stream of nitrogen. High vacuum was utilized to remove the last traces of the solvent. The sample (fraction D) was colorless, extremely viscous and had a peroxide number of 3,185. Peroxide numbers and spectral properties of the fractions are listed in table 2.

Absorption studies. The animals used in these investigations were mature rats[4] which were subjected to thoracic duct cannulation according to the technique of Bollman et al. ('48). Preliminary investigation revealed that pre-operative administration of 2 ml of oxidized soybean oil (PN = 1200) was frequently fatal to the subject. A similar amount of peanut oil was employed, therefore, in the pre-operative procedure. Lymph was then collected for a 20- to 24-hour period at room temperature and was stored at −20°C. These samples served as the source of control lymph fat. After the initial period of lymph collection, a dose of air-oxidized soybean oil was administered to the cannulated rat. Lymph collection was then continued for periods extending up to 12 hours. These samples were also stored at −20°C. Similar volumes of both control and experimental samples were then extracted with a 3:1 mixture of alcohol and ether, the protein removed by centrifugation, and the alcohol-ether layer evaporated in a tared flask under nitrogen on a warm-water bath, for determination of peroxide number. Other lymph fat samples were examined for conjugated diene by dissolving the fat isolated from 2 ml of lymph in 50 ml of ethanol and examining solution at 232 μ with a Carey recording spectrophotometer.

TABLE 2

Peroxide number and conjugated diene concentration in the various fractions of oxidized soybean oil

Fraction	Peroxide number	Meq. conj. diene/kg oil[1]
A	13	0
B	927	1190
C (unseparated)	1156	780
D	3185	1602

[1] The fact that the peroxide numbers and conjugated diene concentrations do not change in parallel fashion probably indicates that the unsaturated centers of many molecules have suffered further attack after the initial peroxide formation.

Xanthine oxidase assay. The intestinal xanthine oxidase of rats was assayed as follows: the animals were decapitated and the upper third of the small intestine excised; intestinal contents were flushed out with approximately 10 ml of saline and 0.5 gm of the upper end of the washed intestinal section placed on dry ice until homogenized; homogenization was conducted at 5°C in 10 ml of an 0.015 M sodium pyrophosphate buffer contained in an all glass homogenizer. The general method of enzyme assay was that of Dhungat and Sreenivasan ('54). Milk xanthine oxidase was purified according to the method of Ball ('39).

RESULTS

Rat growth experiments. In the initial growth study, oxidized oil (1200 PN) was diluted with fresh oil to give peroxide numbers of 800, 400 and 100. Four variations of diet A were prepared: three with each of the diluted oils and the 4th with the original 1200 PN oil. A 5th diet containing fresh oil served as the control. Extraction of the fat in these diets showed that the peroxide number did not change on mixing, after three weeks' storage at −20°C, or on standing at room temperature for three days.

After consuming the control diet for 6 days, 50 weanling rats were divided into 5 groups of 10 each, equally divided between males and females. One of these groups was assigned to each of the diets previously described, and the animals were weighed individually twice a week for the ensuing 70 days. The average growth curves of the female rats in each group are presented in figure 1. The diets containing 20% of fat with peroxide numbers

[4] Sprague-Dawley strain.

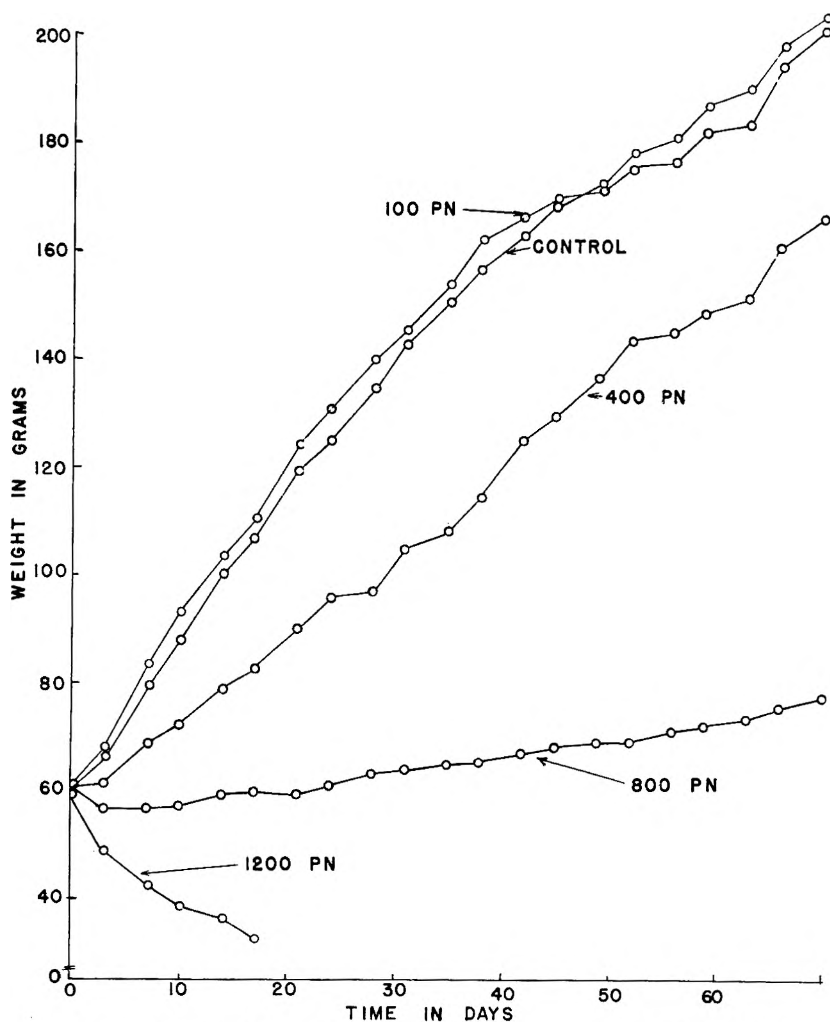

Fig. 1 Growth of female rats receiving various levels of oxidized fat.

of 400 to 1200 caused a reduction in growth rate directly related to the peroxide number. Consumption of the 1200 PN diet caused death within approximately three weeks. The only other symptoms observed in the animals fed this diet were weight loss and severe diarrhea. Animals receiving the 800 PN diet developed moderate to severe diarrhea, but this symptom had subsided somewhat by the 8th week. Similar effects were observed in the growth of male rats fed these diets.

The data obtained in this experiment indicate that the diet containing 100 PN oil has no effect on growth. It seemed conceivable, however, that under conditions of stress a difference between the control and 100 PN diets might become apparent. Accordingly, an experiment was conducted in which 25 rats, 11 males and 14 females, consumed the 100 PN diet for 8 weeks and then were subjected to a whole-body X-irradiation dose of 500 r.[5] A similar group of animals served as concomitant controls. In both the control and 100 PN diets the concentration of brewers' yeast was increased from 5 to 7.5%

[5] Irradiation was carried out using a Picker 250 KV Industrial X-Ray apparatus, with the following factors: 15 ma, 0.28 Cu parabolic, 0.21 Cu inherant and 1 Al; field size 47 cm^2, FOD 55 cm.

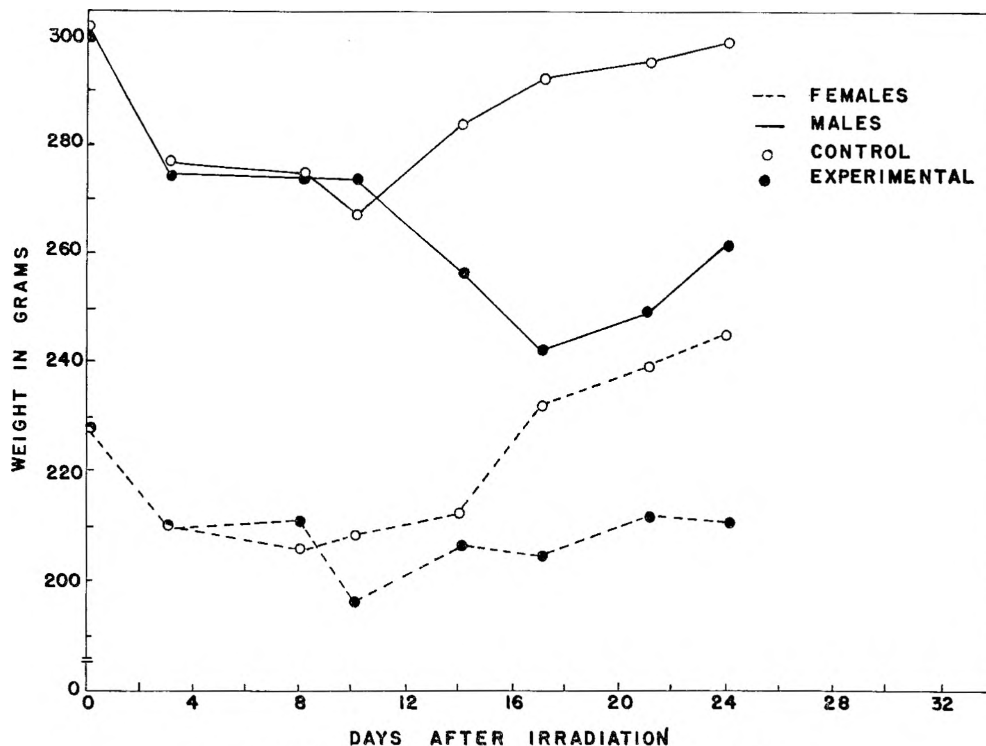

Fig. 2 Effect of diet on recovery from whole body irradiation.

at the expense of sucrose. Figure 2 shows the average group weight changes over the 27-day period following irradiation. The results obtained in this experiment indicate that consumption of the 100 PN diet caused a slower than normal recovery from the stress of irradiation.

In an attempt to compare the effect of the lipid peroxide with that of a simple water-soluble peroxide, and to eliminate diet flavor as a factor, *t*-butyl hydroperoxide, synthesized according to the method of Milas and Surgenor, ('46) was used in both feeding and injection studies. In preliminary injection experiments, 8 mg of this compound in a non-physiological solution was found to be immediately fatal when intravenously administered to rats weighing approximately 400 gm. Intravenous injection of a physiological solution of 1 mg of *t*-butyl hydroperoxide every three or 4 days over a two-week period caused a weight loss and some loss of hair. A growth study was conducted with 4 weanling rats fed control diet A by substituting a solution of 4×10^{-2} M *t*-butyl hydroperoxide for the drinking water. Figure 3 illustrates the average growth of these animals compared with three rats consuming the same diet and drinking tap water. This figure also illustrates the marked loss of weight which occurs when a high-PN diet is substituted for the control diet.

Fractionation experiments. In an attempt to settle the question of whether the toxic principle in oxidized oils is peroxidic or polymeric in nature, the fractions obtained from air-oxidized soybean oil were fed to weanling rats. It was assumed that fraction A contained most of the non-hydroxylated or peroxidized glycerides, while fraction D consisted primarily of glycerides containing fatty acid peroxides. Fraction B was assumed to be a mixture of the two materials. Since incorporation of a highly oxidized oil, namely, fraction D, in the diet makes the food unpalatable, the rats were fed a fat-free diet similar to diet A and the appropriate oil was force-fed. Diet A was modified by increasing the brewers' yeast from 5 to 7.5% and adding

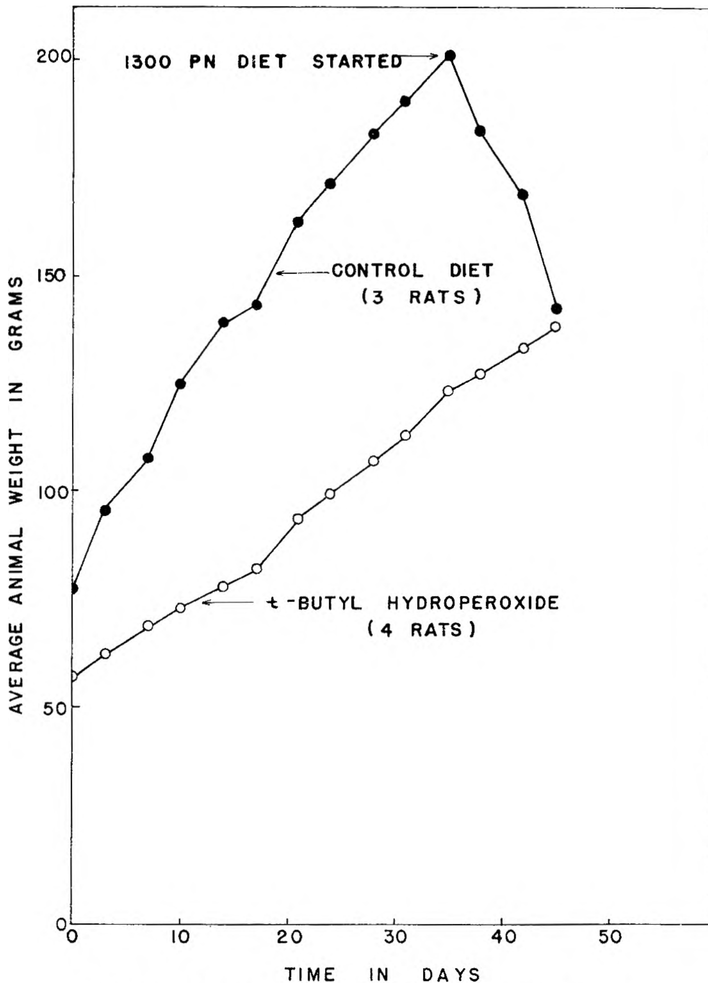

Fig. 3 Growth of rats under several dietary regimes.

2% of guar gum,[6] both at the expense of sucrose. Preliminary considerations indicated that each rat should receive an amount of oil equivalent to 38% of the total amount of calories consumed during the previous 24 hours. Ten grams of the dry diet were weighed into tared glass feed cups for each rat. Two milliliters of water were then mixed with the diet in each cup to make a thick paste which soon hardened and which the animals could eat with a minimum of spillage. An extra cup of diet was prepared each day and used to correct food consumption figures for water evaporation. Fifteen male and 5 female weanling rats ate the fat-free diet for an initial 8-day period. During this period some diarrhea was observed but weight gains were satisfactory and the animals appeared otherwise healthy. At the end of the 8-day period, the male rats were divided into three groups (A, B and C) of 5 each with the female rats comprising a 4th group (D). These group designations correspond to the fraction designations of the oxidized oil. All animals received orally twice the previously described volume of fat-soluble vitamin mix once a week about three hours before any fat was force-fed. If the amount of oil to be administered was more than 0.5 ml, it was given in two equal doses at noon and 4:00

[6] Donated by the Stein-Hall Co., New York.

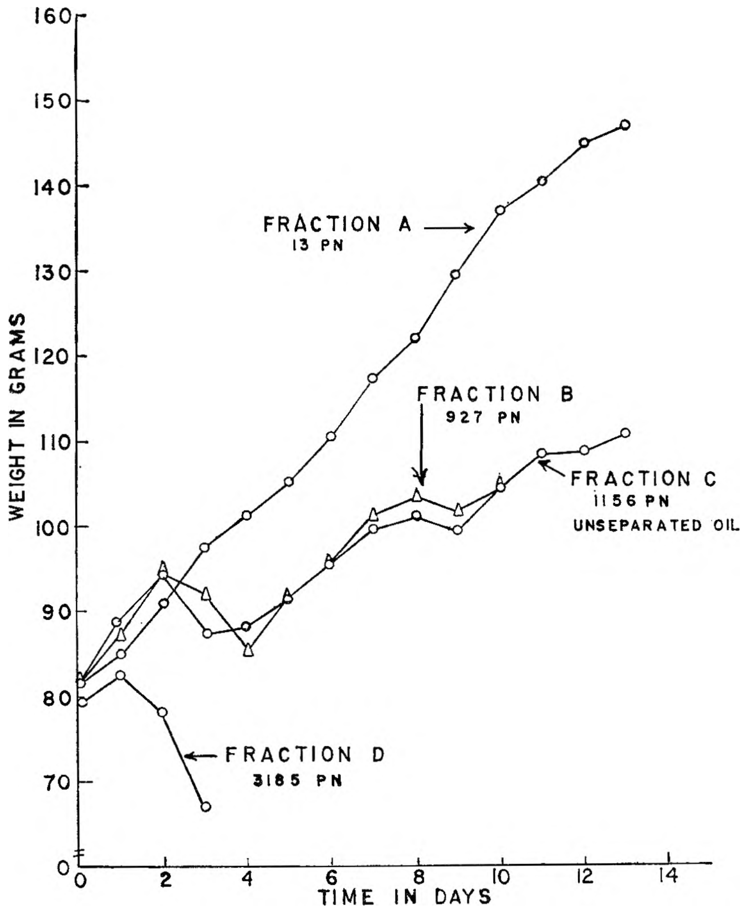

Fig. 4 Growth of rats receiving various fractions of oxidized soybean oil.

P M. The rats were weighed daily during the experimental period. The average weight gains for the 4 diet groups are presented in figure 4.

The rats in group A appeared healthy and ate well in contrast to those in groups B, C and D. Those in groups B and C were indistinguishable in appearance but rats in group D began losing weight after the first day of fat administration. On the third day of the experiment two of the rats in group D were found dead and, since the remaining three appeared moribund, they were killed for pathological examination.

Histopathological examination[7] of these three animals revealed no obvious anatomical basis for their condition. The livers of these rats showed slight enlargement of the nuclei of the parenchymal cells and prominent nucleoli. Moreover, the small intestines of these animals had cytoplasmic vacuoles distending the mucosal walls. Examination of all other organs generally gave negative findings.

Absorption experiments. In order to ascertain the actual site of action of the toxic principle in oxidized fat, it was deemed of primary importance to determine whether any of the peroxide-containing material could be absorbed through the intestinal wall. For this study, rats with thoracic duct cannulae were given small doses of oxidized soybean oil, and the lymph was collected for periods varying between two and 24 hours. After isolation, the lymph fat was analyzed for peroxide and conjugated

[7] Histopathology performed under the direction of Dr. D. Moyer of the Dept. of Pathology, UCLA School of Medicine.

TABLE 3

Peroxide numbers of lymph fat obtained from rats fed either 0.5 to 1.0 ml of air-oxidized soybean oil (PN = 1200) or 2 ml of peanut oil

Rat no.	Oil fed	Lymph extracted	Weight of extracted fat	Peroxide no. of fat
		ml	*mg*	
A	Peanut	4.0	213.7	0.9
	Oxidized soybean	4.0	242.9	32.7
B[1]	Peanut	4.0	188.5	13.1
	Oxidized soybean	4.0	135.0	13.9
C[1]	Peanut	3.0	71.1	4.2
	Oxidized soybean	3.0	161.5	4.3

[1] Rats B and C are presented as typical of the 25 rats in which no significant change in the PN of the lymph fat was found.

diene. The results of the peroxide determinations are shown in table 3. The results of the experiment with rat A led to the conclusion that fatty acid peroxides may be absorbed as such. However, in subsequent experiments involving 25 cannulated rats it has been impossible to detect a significant rise in the peroxide number of lymph fat as the result of feeding oxidized soybean oil with a PN of 1200. The results obtained with rat A were, therefore, assumed to be either an artifact or an exceptional case of absorption.

Ultraviolet examination of a 1:50 dilution in ethanol of lymph fat from cannulated rats yielded the results typical of those presented in table 4. It can be seen that there was an increase in light absorption at 232 mμ (the maximum for conjugated diene) in the lymph of those rats which had been fed the oxidized oil. In the case of rat 6, 3 ml of oxidized oil (PN = 1100) were fed by stomach tube before the cannulation. Collection was begun immediately after the operation (two hours after feeding) and at no time did this animal receive further administrations of oxidized oil. It therefore appears that even though the peroxide itself may not appear in the lymph, its reduction products may be absorbed. The results of the absorption experiments suggest that the fatty acid hydroperoxides are destroyed during or before the absorption process and further, that the toxic effect probably takes place primarily in the intestinal cells.

Table 4

Optical densities at 232 mμ observed in lymph fat from rats fed oxidized soybean oil

Rat no.[1]	Amt. ox. oil fed	Length of collection time	232 mμ O.D. at[2]
	ml	*hours*	
9	0.5–1.0	4.5	0.075
	0.5–1.9	4.0	0.024
6	3.0	21.5	0.030
		5.0	0.130
		3.5	0.021
		16.0	0.058
		8.0	0.008
		16.0	0.000

[1] These results are presented as typical of several experiments performed.

[2] The optical density at 232 mμ (the maximal for conjugated diene) is a measure of the absorption of the fatty acid chain containing the unsaturated center prior to feeding the oxidized oil. The lymph fat had no significant absorption at this point under these conditions of dilution.

Intestinal xanthine oxidase assay. Since the absorption studies had led to the hypothesis that the primary site of toxicity was the intestine, and the pathology of these animals was generally negative, an investigation of the effect of oxidized fat on intestinal xanthine oxidase as a representative intestinal enzyme was undertaken. This enzyme was selected because it is fairly well characterized and is sensitive to lack of a dietary component (McQuarrie and Venosa, '45).

Twelve mature male rats were fed a control diet similar to diet B except that unoxidized soybean oil was substituted for the oxidized oil and the level was increased from 15 to 20% at the expense of sucrose. This control diet was continued for 10 days, at the end of which period the animals were paired on the basis of weight.

TABLE 5

Inhibition of intestinal xanthine oxidase by the ingestion of oxidized fat

Days on experiment	Diet	Feed ingested	Enzyme activity[1]	Intestinal protein[2]	Animal weight	
					Initial	Final
		gm/day	*µl* O_2	%	*gm*	
0	Control	0	23.5	73.9	226.5	
	Control				212.5	
2	Oxidized	2.4	8.0	62.4	211.0	207.0
	Control	2.4	28.5	59.4	201.5	—
5	Oxidized	3.0	1.5	64.5	193.0	178.5
	Control	3.0	4.5	63.6	194.0	180.0
7	Oxidized	3.7	0.5	68.7	212.0	189.0
	Control	3.7	10.0	62.1	204.0	194.0
9	Oxidized	4.9	1.0	66.9	203.0	177.5
	Control	4.9	13.0	63.6	204.0	209.0
12	Oxidized	5.9	0.0	62.1	200.5	188.0
	Control	5.9	25.0	56.7	197.0	203.5

[1] Corrected for endogenous activity.
[2] Determined by method of Lowry et al. ('51).

One pair was killed at this time for enzyme assay. One animal in each of the 5 remaining pairs was randomly assigned to an experimental diet containing 20% of air-oxidized soybean oil (PN = 1200). The control diet was pair-fed for the duration of the experiment. Pairs of rats were killed at intervals of 2, 5, 7, 9 and 12 days after initiation of the experiment and the intestinal xanthine oxidase of each animal assayed. The results of these assays are presented in table 5.

The observed inhibition of intestinal xanthine oxidase appears to be specific since it cannot be due to either lower protein intake or intestinal edema. One possible explanation for this enzyme inhibition is oxidation of sulfhydryl groups as suggested by Potter and DuBois ('43) in their study of succinic dehydrogenase. For an investigation of this possibility a group of 12 weanling female rats were paired and subjected to the same dietary regimen as in the previous experiment. Pairs of rats were killed each day for 6 days and the levels of SH in each intestine determined. The intestinal samples were prepared as described above and the method of Grunert and Phillips ('51) was used to determine the levels of SH, reported as glutathione. The results obtained in this experiment are given in table 6.

Apparently, the inhibition of intestinal xanthine oxidase is not directly connected with oxidation of sulfhydryl groups. In order to investigate further the mechanism of this enzyme inhibition, milk xanthine oxidase was purified by the method of Ball ('39) and *t*-butyl hydroperoxide was used as the inhibitory compound. The assay

TABLE 6

Levels of intestinal glutathione in weanling female rats consuming either oxidized or unoxidized soybean oil

Rat no.	Oil	No. of days on experiment	Intestinal glutathione level
			µg/mg fresh tissue
7	Control	1	0.38
8	Oxidized		0.57
9	Control	2	0.51
10	Oxidized		0.75
23	Control	3	0.27
24	Oxidized		0.21
11	Control	4	0.24
12	Oxidized		0.37
19	Control	5	0.19
20	Oxidized		0.22
25	Control	6	0.27
26	Oxidized		0.43

TABLE 7

Warburg assay of the inhibition of milk xanthine oxidase by t-butyl hydroperoxide

Flask components						Activity[4]
Buffer[1]	Enzyme	*t*-Butyl hydroperoxide 1.1×10^{-4} M	FAD[2,3] 1 mg/ml	Xanthine 1 mg/ml	H_2O	O_2 uptake: 60 min. totals
ml	*ml*	*ml*	*ml*	*ml*	*ml*	*µl*
1.0	0.3				1.7	0.0
1.0	0.3			1.0	0.7	80.3
1.0	0.3	0.1		1.0	0.6	73.9
1.0	0.3	0.1	0.1	1.0	0.5	90.6

[1] 0.039 M K–$NaPO_4$, pH 7.54.
[2] Flavin adenine dinucleotide.
[3] Obtained from California Corporation for Biochemical Research, Los Angeles. Reported absorption ratio at pH 7.0, 264 mµ/450 mµ = 3.22.
[4] Average of duplicate flasks.

TABLE 8

Warburg assay of the inhibition of rat intestinal xanthine oxidase by t-butyl hydroperoxide

Flask components						Activity[1]
Buffer[2]	Enzyme[3]	*t*-Butyl hydroperoxide 1.1×10^{-4} *M*	FAD[4,5] 1 mg/ml	Xanthine 1 mg/ml	H_2O	O_2 uptake: 60 min. totals
ml	*ml*	*ml*	*ml*	*ml*	*ml*	*µl*
1.0	0.5				1.5	43.6
1.0	0.5			1.0	0.5	89.1
1.0	0.5	0.1		1.0	0.4	42.4
1.0	0.5	0.1	0.1	1.0	0.3	71.7

[1] Average of duplicate flasks.
[2] 0.039 M K–$NaPO_4$, pH 7.54.
[3] A 5% intestinal homogenate.
[4] Flavin adenine dinucleotide.
[5] Obtained from California Corporation for Biochemical Research, Los Angeles. Reported absorption ratio at pH 7.0, 264 mµ/450 mµ = 3.22.

technique was essentially the same as that previously described except that a 0.039 M phosphate buffer with equal concentrations of sodium and potassium at pH 7.5 gave higher oxygen uptake and was, therefore, substituted for the pyrophosphate buffer. In view of the work of Bernheim, et al. ('52) and Ottolenghi et al. ('55) the effect of flavin adenine dinucleotide (FAD) on the inhibited enzyme was investigated. The data obtained in this experiment are reported in table 7.

The results of this experiment indicate that *t*-butyl hydroperoxide is capable of inhibiting milk xanthine oxidase and further, that this inhibition may be reversed by FAD. The high value for the flask containing FAD probably means that the enzyme preparation is somewhat deficient in FAD. A similar assay using a 5% intestinal homogenate from a stock rat as the enzyme source yielded the results presented in table 8.

This experiment corroborates the similarity of the inhibitory effects of the hydroperoxide and the ability of FAD to reverse these effects although apparently not as completely as with the purer preparation.

DISCUSSION

The investigations described above support earlier work in establishing that the toxicity ascribed to air-oxidized oils correlates well with the fatty acid peroxide content of these materials. The feeding experiment in which *t*-butyl hydroperoxide was utilized substantiates, at least in some degree, the toxicity of fatty acid peroxides, although this substantiation must be qualified because of differences in structure

and probable mode of absorption. Nevertheless, from the data obtained in this experiment it can be calculated that the consumption of peroxide in the form of *t*-butyl hydroperoxide corresponds to a diet containing 20% of oil with a PN of approximately 600. It is interesting to note that the average growth curve of these animals falls between those for rats receiving diets with oils of 400 and 800 PN. The fractionation of air-oxidized soybean oil showed predominantly polymers of a polar nature, probably polyperoxides, and little or no polymers of the type found in heat-oxidized oil. This study also illustrates the complexity of the reactions occurring in the autoxidation of an oil, since if a simple generation of peroxide groups were the only reaction occurring, equivalence between peroxide oxygen and conjugated diene should be observed. Such is not the case, however (table 2). Although the work reported here demonstrates the toxicity of oxidized oil to be due entirely to peroxide formation, we have not investigated the toxic principle of anaerobically heated oils. Thus, it may be suggested that two separate classes of toxic compounds are possible in autoxidized oil—fatty acid peroxides when the fat is oxidized in air at only slightly elevated temperatures for 1.5 weeks or less and fatty acid polymers when the oil is heated to temperatures in the neighborhood of 250°C with the exclusion of oxygen for periods of one to two days. The conditions of autoxidation for a maximum yield of one class or the other will vary, however, with different oils. In our laboratory we have observed that extended aeration of soybean oil results in an oil of lower than expected peroxide content, and that the application of high temperatures at any point during the autoxidation process will have the same effect. It is probable that either of these modifications tends to decrease the peroxide content of the oil by formation of degradation products and polymers.

The primary site of toxicity, as demonstrated in the cannulated rats, appears to be the intestine. The gross observations and the limited pathology data available appear to support this conclusion. Even the irradiation experiment lends some indirect support to this hypothesis since it is known that the intestine is one of the most sensitive organs to whole-body irradiation, and extended recovery periods were observed in those animals receiving a dietary fat containing peroxide.

The mechanism or mechanisms by which fatty acid peroxides cause the observed toxic effects still need clarification and amplification. In the absence of observable gross changes in any of the organs examined, it seems likely that the injury is of a more subtle nature. The inhibition of intestinal xanthine oxidase, however, points to the possibility that choline oxidase and amine oxidase may be inhibited in a similar manner and that succinoxidase and cytochrome oxidase may also be inhibited but in a manner as yet unknown (Bernheim et al., '52; Ottolenghi et al., '55).

SUMMARY

The toxicity of air-oxidized soybean oil to weanling rats was investigated in several aspects.

Growth studies demonstrated that the concentration of the toxic principle corresponded closely to the peroxide concentration of the oil. Moreover, separation of the oxidized oil into high-, medium- and low-peroxide fractions revealed that toxicity again followed peroxide concentration.

Histopathological examination of rats fed oxidized soybean oil gave generally negative findings but indicated that the intestine might be involved. Moreover, absorption of fatty acid peroxides in mature rats, studied by means of thoracic duct cannulation, indicated that although the reduced products of the peroxides were absorbed, the peroxides themselves were destroyed in the intestine and probably had their action at that site. Recovery from the effects of whole-body irradiation, a condition also affecting the intestine, was delayed by diets containing fat with peroxide numbers as low as 100.

Inhibition of intestinal xanthine oxidase by air-oxidized soybean oil and its reversal by exogenous flavin adenine dinucleotide suggest that the specific toxicity of the lipid peroxides may be at the level of the intestinal enzymes.

ACKNOWLEDGMENTS

The authors wish to acknowledge the technical assistance of Mary L. Gouze, Annette Terzian, Joan Hillsley and Loren Garretson.

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The Growth, Breeding and Longevity of Rats Fed Irradiated or Non-Irradiated Pork[1,2]

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The age-old problem of food preservation has received a new impetus with the use of ionizing radiation. Although many problems exist, the practical use of this method has a promising future.

The research of Poling and coworkers ('55) failed to demonstrate any "unwholesomeness" in diets of rats fed irradiated beef, although some loss of vitamin E was noted. Cathode or beta rays were used in the irradiation process. A similar study was reported by Bubl and Butts ('56) using mixed organ meats subjected to gamma irradiation. No toxic effects were noted in breeding performance or longevity of the rats. The nutritional value of irradiated synthetic diets was studied by Richardson and Brock ('58). Reproduction and longevity were measured. They concluded that there was a slight difference in favor of the non-irradiated diet, but the difference was so small that they did not consider it to be of any practical importance. In a short-term experiment Read and coworkers ('58) reported no toxicity in 14 irradiated foods when fed to rats for 8 to 12 weeks at 35% of the dry weight of the diet.

The work reported here is part of the contractual program of the Office of the Surgeon General, Department of the Army, to determine the wholesomeness of irradiated foods, based on the procedure recommended by Lehman and Laug ('54). This broad research program was established to test a wide spectrum of representative foods from which extrapolation to most others could occur. Pork was one of these foods as it represented a fresh meat of high lipid and high moisture content.

The long-term feeding experiments were designed to obtain data concerning food consumption, growth, reproduction, lactation, size and viability of young and longevity. Four direct-line generations of rats were used in obtaining the data reported here.

EXPERIMENTAL

Both the irradiated and non-irradiated pork used in these experiments was supplied by the Quartermaster Food and Container Institute, Chicago. The cuts used were boned loin or shoulder which had been minced in a mechanical grinder, then passed through a fine-plate sausage grinder. The mass was then thoroughly mixed, usually in quantities of 1000 pounds or greater. The mixture was then packed into no. 2 (307 by 405) "C" enamel cans, sealed under vacuum and sharp frozen. The cans of pork serving as the control (non-irradiated) were shipped to this station frozen and stored at −10°F. until needed.

Two levels of irradiation were used: 2.79 megarad (3 megarep.) and 5.58 megarad (6 megarep.) to prepare the experimental meat. These levels were obtained in the canal of the Materials Testing Reactor, Phillips Petroleum Company, Idaho Falls, Idaho, using spent fuel rods giving mixed gamma radiation. Upon completion of irradiation, the cans of pork mixture were shipped to this station at ambient temperature. They were stored at room temperature, 78°F. (range 72 to 84°F.), for from three to 8 months from the date of irradiation prior to mixing in the diet.

Both the irradiated and control pork were cooked prior to mixing in the ration.

Received for publication April 20, 1959.

[1] This paper reports research undertaken in cooperation with the Office of the Surgeon General, Department of the Army, under Contract DA-49-007-MD580.

[2] Technical paper no. 1215, Oregon Agricultural Experiment Station.

The method used simulated conditions used in actual practice in the home or in the mess hall. The contents of two similar cans were emptied into a shallow aluminum pan having a tight-fitting, slip-on cover, in order to cut down loss of moisture and volatile components and avoid the formation of a crust. Pork was baked at 350°F. for 75 minutes. The pans and their contents were cooled slowly to room temperature without removing covers. The cooked meat was then weighed in portions for mixing into the ration, shown in table 1.

TABLE 1
Diet of animals on pork experiment

	Amount	
	As prepared	%
	gm	
Cooked pork meat (Control or irradiated)	1316	35[8]
Lactalbumin[1]	200	10
Salts[2]	80	4
Celluflour[3]	100	5
Inositol	4	0.2
Vitamin pre-mix[4]	2	0.1
Choline chloride	6	0.3
Liver extract[5]	10	0.5
Guar gum[6]	40	2.0
Potato starch[7]	858	42.9
Total		100.0

[1] Whitson Products, Boscabel, Wisconsin.
[2] Jones and Foster mix, Nutritional Biochemicals Corporation (Jones and Foster, '42).
[3] Celluflour, obtained from Chicago Dietetic Supply House, Chicago.
[4] The vitamin mix contained the following in grams: thiamine 4, riboflavin 8, pyridoxine 4, niacin 4, calcium pantothenate 20, *p*-aminobenzoic acid 240, menadione 16, potato starch to make 700 gm.
[5] Wilson's 1:20, Wilson and Company, Chicago.
[6] Ja*guar* A-20-A, Stein Hall and Company, New York.
[7] Idaho Potato Starch Company, Blackfoot, Idaho.
[8] As dry solids.

Except for Saturdays and Sundays, the ration was mixed daily and kept refrigerated (40°F.) until fed—rarely more than three days. The fat-soluble vitamins were fed twice a week using a calibrated medicine dropper. The levels obtained for each dose were a minimum of 50 U.S.P. units of vitamin A, 5 U.S.P. units of vitamin D and 9 mg of DL, α-tocopherol (9.9 I.U.).

First generation rats (150) were weanlings raised at this station from our highly inbred Wistar Strain stock colony. The animals of subsequent generations were the offspring of the control or experimental groups of the first, second or third generations. The pattern of selection for the first (parent) generation was as follows: 6 animals (three males, three females) having weights at weaning as close as possible were selected from a single litter. These brother-sister pairs were distributed among three groups, namely, control, 1X, and 2X, a male and a female in each. This grouping was replicated 25 times by the use of 25 sets of litter-mates, the replications being set up over a three-month period. All animals were 28 days old at the start of the period of measured growth.

The animals were allowed food and water ad libitum and housed individually in an air-conditioned area. Animal weights were recorded each 5th day for the first-generation growth periods, each 7th day in the subsequent generations. Growth of the first-generation groups was recorded for the first 100 days of experiment. At the end of this period, the females of all groups were bred. Each female was mated with at least three different males on the same dietary regimen, each male being housed with the female for a 6-day period before being moved to another female. At the end of three rotations (18 days), the female's weight was recorded as she was transferred to a nesting cage. She was allowed to remain alone until parturition, or until 25 days after removal from last male.

The date of birth and number of young born were recorded following parturition, including stillborn pups. Within two days following birth, those litters of over 8 were randomly culled to 8 and the young were weaned at 28 days. When the first litters reached weanling age, the pups were weighed, examined carefully for abnormalities and the sex distribution recorded. Abnormal animals were examined by the pathologist for gross or histopathological changes. The others were discarded.

The females were rested for two to three weeks after weaning their pups and then bred again as above. Females which did not produce a litter on either of these two

trials were considered sterile and not used again.

RESULTS

The data obtained during the 14-week growth period for the first, second and third generations are shown in tables 2 and 3. The analysis of variance of this data showed no significant differences between treatments or generation and treatments.

The breeding phases of the experiment are shown in tables 4, 5 and 6. Statistical analysis again showed no significant difference between treatments. However, some generation differences are obvious. In second and third generations the weaning weight of both pups and mothers were, in

TABLE 2

Weight average of each group of 25 animals receiving pork, either frozen or held at room temperature after irradiation

(First generation)[1]

Time	Control 25/group		2.79 megarad 25/group		5.58 megarad 25/group	
	Males	Females	Males	Females	Males	Females
days						
0	64	60	61	63	63	60
10	109	95	104	93	107	94
20	165	138	160	138	163	138
30	210	160	201	158	208	162
40	246	179	269	180	260	177
50	276	194	300	195	291	189
60	304	205	302	203	306	205
70	327	216	326	213	315	215
80	338	221	338	218	339	220
90	350	225	348	221	340	223
100	362	230	366	227	346	230

[1] Analysis of variance:

Source of variation	D.F.	F value
Treatments	2	2.03
Sex × treatments	2	0.65
Generation × treatments	4	1.33
Sex × generation × treatments	4	0.53

TABLE 3

Weight average of each group of 25 animals receiving pork, either frozen or held at room temperature after irradiation

(Second and third generation)[1]

Generation Dose in megarad	Males						Females					
	2			3			2			3		
	0	2.79	5.58	0	2.79	5.58	0	2.79	5.58	0	2.79	5.58
weeks												
0	48	42	44	70	65	61	48	42	41	67	63	59
1	65	59	66	96	90	85	63	66	63	90	86	83
3	129	124	128	169	160	161	112	111	109	141	137	138
5	198	185	186	231	222	224	148	147	145	172	169	167
7	257	250	250	279	274	274	175	177	174	193	195	190
9	294	290	291	315	305	313	190	195	190	209	213	207
11	325	319	322	337	341	340	202	207	201	220	230	217
13	349	349	350	351	354	360	210	219	211	228	231	225
14	358	360	360	360	364	368	216	225	219	229	235	232

[1] Analysis of variance:

Source of variation	D.F.	F value
Treatments	2	2.03
Sex × treatments	2	0.65
Generation × treatment	4	1.33
Sex × generation × treatments	4	0.53

TABLE 4

Reproduction records of first generation pork-fed animals. Control pork held in frozen state until mixed in diet. Irradiated pork held at room temperature. Same treatment was used in second and third generations

Litter	Control		2.79 megarad		5.58 megarad	
	1	2	1	2	1	2
No. females bred	25	25	26	25	26	26
No. producing a litter	21	20	24	22	23	22
No. weaning all pups[1]	16	12	16	17	18	19
No. weaning less than all:						
1 pup	2	6	1	2	3	2
2 pups	0	1	3	0	1	0
3 or more pups	1	1	2	0	1	1
No. weaning no pups	2	0	2	3	0	0
No. of sterile females	4	5	2	2	3	4
Av. weight in gm, pups at weaning[2]	47.8	46.8	50.8	40.4	46.1	39.8
Range	(39–68)	(30–60)	(36–68)	(31–56)	(26–68)	(27–60)
Av. weight in gm, mothers at weaning	228	220.5	227.5	223.0	219.0	224.1
Range	(194–260)	(151–248)	(196–277)	(194–255)	(195–280)	(197–260)
Total pups born	191	172	217	218	225	205
Total stillborn	12	3	0	2	1	0
Total culled out	27	27	33	44	45	30
Total weaned	(142) ♂ 71 ♀ 71	(129) ♂ 58 ♀ 71	(154) ♂ 77 ♀ 77	(148) ♂ 74 ♀ 74	(171) ♂ 88 ♀ 83	(160) ♂ 85 ♀ 75
Total not surviving to weaning	10	13	30	24	8	6

[1] Litters over 8 culled randomly to 8.
[2] Weaning at 28 days.

TABLE 5

Reproduction records—pork-fed animals
(Second generation)

Litter	Control		2.79 megarad		5.58 megarad	
	1	2	1	2	1	2
No. females bred	24	23	25	24	23	23
No. producing a litter	21	19	24	23	22	23
No. weaning all pups[1]	19	17	18	21	17	21
No. weaning less than all:						
1 pup	1	2	2	1	0	1
2 pups	0	0	0	0	3	0
3 or more pups	0	0	1	0	0	0
No. weaning no pups	1	0	3	1	2	1
No. sterile females	3	2	1	1	1	0
Av. weight in gm, pups at weaning[2]	59.9	66.5	60.0	67.1	60.8	62.1
Range	(37–80)	(51–82)	(47–80)	(46–82)	(44–83)	(44–84)
Av. weight in gm, mothers at weaning	232	234	240	259	244	243
Range	(201–264)	(191–271)	(202–269)	(216–286)	(198–291)	(214–281)
Total pups born	186	202	224	253	206	244
Total stillborn	1	2	11	23	6	3
Total culled out	37	54	42	67	45	61
Total weaned	(139) ♂ 67 ♀ 72	(144) ♂ 70 ♀ 74	(155) ♂ 72 ♀ 83	(161) ♂ 79 ♀ 82	(149) ♂ 70 ♀ 79	(169) ♂ 89 ♀ 80
Total not surviving to weaning	9[3]	2	16	2	6	11[4]

[1] Litters culled to 8 at random.
[2] Weaning at 28 days.
[3] Eight pups destroyed when mother found dead on 16th day.
[4] Female died three days after birth; her 10 pups destroyed.

TABLE 6

Reproduction records—pork-fed animals

(Third generation)

Litter	Control		2.79 megarad		5.58 megarad	
	1	2	1	2	1	2
No. females bred	23	22	25	23	25	24
No. producing a litter[1]	22	21	24	23	24	23
No. weaning all pups	19	18	19	20	23	21
No. weaning less than all:						
1 pup	2	0	4	2	1	2
2 pups	0	1	0	1	0	0
3 or more pups	1	0	0	0	0	0
No. weaning no pups	0	2	1	0	0	0
No. sterile females	1	1	1	0	1	1
Av. weight in gm, pups at weaning[2]	58.8	53.1	60.1	56.2	59.6	56.5
Range	(45–70)	(40–61)	(48–76)	(36–71)	(40–79)	(48–76)
Av. weight in gm, mothers at weaning	233	238	248	255	225	246
Range	(198–299)	(201–287)	(219–288)	(216–304)	(174–267)	(190–298)
Total pups born	234	213	256	225	230	236
Total stillborn	4	10	1	7	1	5
Total culled out	58	51	66	46	51	58
Total weaned	(163) ♂ 82 ♀ 81	(141) ♂ 71 ♀ 70	(177) ♂ 83 ♀ 94	(168) ♂ 79 ♀ 89	(177) ♂ 92 ♀ 85	(171) ♂ 80 ♀ 91
Total not surviving to weaning	9	11	12	4	1	2

[1] Litters over 8 culled randomly to 8.

[2] Weaning at 28 days.

general, superior. But in these generations the variance concerning treatment was not significant.

Analysis of variance[3]

Source of variation	D.F.	F value
Av. wt. of pups at weaning		
Treatment	2	0.27
Generation	2	28.60[5]
Treatment × generation	4	0.32
Av. wt. of mother at weaning		
Treatment	2	3.75
Generation	2	11.55[5]
Treatment × generation	4	1.03
Weaning efficiency = $\frac{\text{Total weaned}}{\text{Total weaned + total not surviving}}$		
Treatment	2	5.16[4]
Generation	2	6.65[4]
Treatment × generation	4	3.02

[3] Snedecor, '46.
[4] Significance at 5% level.
[5] Significance at 1% level.

There was a significant drop in weaning efficiency in the first generation of the 2.79 megarad group. This can be seen from the data in table 4, and in the statistical analysis of the data.

Generation	Control	2.79 megarad	5.58 megarad	Generation means
1	0.9210	0.8485	0.9595	0.9097
2	0.9625	0.9470	0.9500	0.9532
3	0.9380	0.9570	0.9910	0.9620
Treatment means	0.9405	0.9175	0.9668	0.9516

Generation or treatment L.S.C.

$$\sqrt{\frac{2(0.00708)}{6}} \times 2.262 = 0.0347$$
$$\times 3.250 = 0.0499$$

In subsequent generations, with the same treatment, there was no depression. We have found no apparent explanation for this depressed weaning performance. We also feel it has little significance in the outcome of the entire experiment.

Table 7 gives the average survival time in days for 150 animals of the parent generation. The animals surviving were sacrificed at 104 weeks (730 days). The statistical analysis of the survival data showed no significant differences between treatments.

In addition to the material presented above, a large amount of other data has been accumulated. Food intake for each animal during the growth period showed no differences between treatments. However, the over-all food intake increased with generation, the third generation eating 50% more than the first generation.

In the longevity group there were no treatment differences as measured by weight and food intake at 12, 18 and 24 months of age.

Hematological examination of a selected group of animals of each sex and treatment was carried out. Red cell, white cell and differential blood count data are available. No differences between treatments were demonstrated.

SUMMARY

Pork irradiated at either 2.79 megarad or 5.58 megarad and held at room temperature, or control pork which was non-irradiated but was held in the frozen state, was fed ad libitum to rats over a 4-generation, two-year study. The pork was fed as 35% of the dry weight of the diet. The remainder of the diet was non-irradiated and was considered nutritionally adequate.

TABLE 7

Longevity of first-generation animals, ingesting frozen pork or pork held at room temperature after two levels of irradiation

Food dosage	Animals surviving 730 days		Average survival time	
	Males[1]	Females[1]	Males[1]	Females[1]
megarad			*days*	*days*
0	12	15	624	619
2.79	16	12	630	606
5.58	13	8	608	625

[1] Twenty-five per group.

No statistical differences were obtained in growth, breeding and longevity among any of the groups.

ACKNOWLEDGMENT

The authors would like to acknowledge the technical assistance in these feeding studies of Mrs. Margaret Millsap, Mrs. E. Craig, Mrs. Lois Toner and Mrs. Mary Thompson.

We should also like to thank Stein Hall and Company, New York, for the very generous supply of Ja*guar* (guar gum) so useful in our diets.

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Studies of the Effect of Heat Treatment on the Metabolizable Energy Value of Soybeans and Extracted Soybean Flakes for the Chick

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The beneficial effect of moist heat on the nutritive value of soybeans and soybean-oil meal for chickens, rats and other monogastric species is well established. A major part of the beneficial effect is due to the inactivation of one or more trypsin inhibitors (Borchers, et al., '48; Westfall and Hauge, '48). For the rat at least, the destruction of another heat-labile substance (soyin) is also necessary (Liener, '53).

The major criteria which have been used to evaluate the effects of heat treatment are growth rate of experimental animals and the rate and extent of protein digestion *in vivo* and *in vitro*. In the chick, the digestibility of the protein of raw soybeans or soybean-oil meal is only about 80% that of heated beans or meal (Evans et al., '47; Bouthilet et al., '50). In contrast, relatively little difference in digestibility between raw and heated soybean protein has been found in the rat (Osborne and Mendel, '17; Hayward et al., '36), although rate of digestion appears to be slower for raw protein (Carroll et al., '52). Supplementing the diet with trypsin (Almquist and Merritt, '52, '53) or crystalline amino acids (Fisher and Johnson, '58) has been shown to overcome the growth retarding effect of raw soybean-oil meal for chicks, indicating that the presence of trypsin inhibitors may account adequately for its poor nutritive value.

The determination of metabolizable energy provides a means for determining the over-all utilization of a diet or diet component. It measures the metabolic sum of the processes of digestion, absorption and utilization of a diet in energy units (Hill and Anderson, '58; Anderson et al., '58). While it has the disadvantage of not identifying the nature or source of the products of excretion, this procedure has the advantages of convenience (particularly for avian species) and of avoiding the need for determining individual substances or groups of substances in the excreta. In other work in our laboratories, metabolizable energy has been found to be a highly precise measure of dietary energy value for the chicken, and a useful tool in quantitative studies of energetic relationships in growth.

It was decided to apply this method to the quantitative evaluation of the effects of heating on soybeans and extracted soybean flakes as a measure of over-all utilization. Evans and coworkers ('47) had shown fairly good correlation between protein digestibility and growth-promoting value of a series of soybean-oil meals, but no information is available on the effects of heating on the digestion and absorption of other components of soybeans. It was possible, therefore, that heating conditions optimal for protein digestibility might differ from those needed for maximal utilization of other components of the soybean. Furthermore, since most previous work has used rate of growth and its efficiency in relation to protein intake as criteria of nutritional value, it is important to know whether the heating conditions which are optimal according to these criteria are also optimal for greatest utilization as measured by metabolizable energy.

Received for publication September 4, 1959.

MATERIALS AND METHODS

Male cross-bred (RIR × BPR) chicks were used in the experiment and were maintained in electrically-heated, thermostatically-controlled battery brooders with raised wire screen floors in a temperature-controlled laboratory. Ten chicks were randomly assigned at one day of age to each experimental group, and two duplicate groups were fed each of the experimental diets. Feed and water were supplied ad libitum.

The composition of the experimental diets is shown in table 1. One of the diets was based on the use of ground soybeans as the major source of protein. The other diet was based on the use of extracted dehulled soybean flakes and degummed soybean oil to supply the same amount of protein and fat as that supplied by the ground soybeans, with the addition of cellulose to simulate the hull of the soybean and to equalize the formula weight on a dry matter basis. The soybeans and extracted dehulled soybean flakes had the following percentage composition, expressed on a dry matter basis: soybeans, protein 43.5; fat 20.3; crude fiber 6.8; ash 4.7; extracted dehulled flakes, protein 56.4; fat 0.9; crude fiber 2.7; ash 5.7. Both diets were formulated to contain adequate amounts of all known nutrients required by the chick, and were liberally supplemented with corn distillers' solubles, dried whey, and fish solubles as sources of unidentified nutrients. On the basis of previous studies with soybean products (Hill and Renner, '60, and unpublished data) the diets were expected to contain approximately 3.5 Cal. of metabolizable energy per gram of dry matter. The protein level of the diets was formulated to supply one gram of protein (N × 6.25) for each 13 Cal. of metabolizable energy, a level adequate to meet the needs of the rapidly growing chick. DL-Methionine was

TABLE 1
Composition of experimental diets

Component	Soybean flakes diet	Soybean diet
	%	%
Glucose[1]	29.51	29.51
Ground soybeans	—	57.61
Extracted dehulled soybean flakes	44.46	—
Soybean oil (degummed)	11.31	—
Cellulose	1.84	—
Constant ingredients:		
Corn distillers' solubles	2.00	
Dried whey	2.00	
Fish solubles (dry basis)	0.50	
Corn oil	1.00	
DL-Methionine	0.15	
Glycine	0.50	
Limestone	1.83	
Dicalcium phosphate	1.30	
Salt	0.60	
$MnSO_4$	0.03	
Vitamin mixture[2]	0.80	
Mineral mixture[2]	1.15	
Antioxidants (BHT + DPPD)	0.02	
Chromium "bread"[3]	1.00	
Total	100.00	

[1] Cerelose.

[2] Vitamin and mineral mixtures supply, in milligrams per 100 grams of diet: KH_2PO_4, 867; $MgSO_4$, 242; $FeSO_4 \cdot 7H_2O$, 27.8; $ZnCl_2$, 10.5; $Na_2MoO_4 \cdot 2H_2O$, 0.83; $CuSO_4 \cdot 5H_2O$, 0.78; NaI, 0.26; $CoCl_2 \cdot 6H_2O$, 0.17; Na_2SeO_3 0.022; thiamine, 0.5; riboflavin, 1.0; calcium pantothenate, 2.5; pyridoxine, 1.0; niacin, 3.0; folacin, 0.2; menadione, 0.1; biotin, 0.04; vitamin B_{12}, 0.001; choline chloride, 900; USP units vitamin A, 1000; IU vitamin D_3, 150; α-tocopheryl acetate, 2.4.

[3] Contains 30% Cr_2O_3.

added to bring the total sulfur amino acid content of the diets to approximately 3.6% of the protein.

The ground soybeans and the extracted dehulled soybean flakes were subjected to graded levels of heat treatment. Each material was adjusted to approximately 20% moisture content by the addition of water, and held overnight under refrigeration in a closed container to allow uniform moisture distribution. The material was then autoclaved at 107°C (approximately 4 pounds steam pressure) for 10, 40 or 60 minutes in tinned metal trays in shallow layers, approximately 1/4 to 3/8″ deep. Autoclaving for two hours at 15 pounds pressure was used to produce an overheated meal. Subsequent to autoclaving, the materials were dried in a forced-draft oven at 50°C for approximately 18 hours and then allowed to equilibrate in moisture content with the atmosphere prior to grinding.

Data on growth and feed consumption were taken at weekly intervals. In addition, during the fourth week of the experiment, excreta were collected from each experimental lot at 24-hour intervals on 4 successive days for the determination of metabolizable energy. Chromic oxide was incorporated in each of the diets at a level of approximately 0.3% as an index substance in order to eliminate need for quantitative collection of the excreta and quantitative measurements of feed intake. The methods of processing excreta, conducting chemical analyses for moisture, nitrogen, combustible energy and chromic oxide, and computing metabolizable energy from these data have been described previously (Hill and Anderson, '58; Hill et al., '60).

The apparent absorbability of dietary fat was determined by appropriate analysis of feed and excreta. Dietary fat was determined by ether extraction; fecal fat and fatty acids were determined by a slight modification of the method of Fowweather and Anderson ('46). The concentrations of chromic oxide in diets and feces were used to establish the amount of excreta per unit of diet, and absorbability was computed from the fat content of equivalent amounts of diet and excreta without correction for endogenous fat.

RESULTS AND DISCUSSION

Summarized in table 2 are data showing the effect of the various heating treatments on the growth-promoting value and metabolizable energy of extracted, dehulled soybean flakes. Optimal heat treatment as indicated by the rate and efficiency of chick growth during the 4-week assay period was achieved by 10, 40 and 60 minute heating at 107°. The differences between these treatments were not statistically significant according to analysis of variance (Snedecor, '56) and application

TABLE 2

Effect of heat treatment on growth promoting value and metabolizable energy of extracted soybean flakes

Heat treatment of flakes	Growth data, 4 weeks				Metabolizable energy of flakes	
	Average weight		Gain/gm feed			
	gm		*gm*		*Cal./gm dry matter*	
None (raw flakes)	319		0.392		1.52	
	299	*309*[1]	0.386	*0.389*	1.61	*1.56*
10 minutes, 4 pounds	462		0.650		2.56	
	451	*457*	0.645	*0.648*	2.69	*2.62*
40 minutes, 4 pounds	475		0.636		2.57	
	466	*470*	0.636	*0.636*	2.63	*2.60*
60 minutes, 4 pounds	453		0.625		2.52	
	449	*451*	0.621	*0.623*	2.51	*2.51*
120 minutes, 15 pounds	401		0.502		2.09	
	387	*394*	0.505	*0.504*	1.96	*2.02*

[1] Figures in italics are averages for duplicate lots; individual lot data in left column for each measurement.

of the Duncan multiple range test (Federer, '55). The unheated flakes and the overheated flakes promoted significantly less rapid and less efficient growth. The metabolizable energy of the heated flakes was calculated from the metabolizable energy of each complete diet by subtracting from it the values for glucose, soybean oil and the constant diet ingredients determined in previous work (Anderson, et al., '58, and unpublished data). The calculated value of these ingredients per gram of diet dry matter was 2.39 Cal. The data in table 2 show that the samples of soybean flakes which promoted most rapid and most efficient growth also contained the highest metabolizable energy value per gram; the differences between 10-, 40- and 60-minute heating at 107°C were not significant.

Summarized in table 3 are similar data on rate and efficiency of growth and metabolizable energy value for the various samples of soybeans. All of the soybean samples heated at 107°C (10, 40 and 60 minutes) had essentially equal value as measured by rate and efficiency of growth; the differences between them were not statistically significant. The metabolizable energy of the variously heated soybean samples was estimated by subtracting from the metabolizable energy of the complete diets the previously determined values of the other ingredients; by calculation, the other ingredients contributed 1.34 Cal. of metabolizable energy per gram of diet dry matter. Maximal metabolizable energy value was obtained with the sample heated for 10 minutes at 107°C; it was significantly ($P < 0.05$) higher than the samples heated 40 and 60 minutes. The sample heated for 10 minutes also tended to be the most efficient for promoting chick growth. The samples heated for 40 and 60 minutes had metabolizable energy values significantly greater than the unheated and overheated soybeans.

In previous work of this laboratory, data have been obtained on the metabolizable energy content of various soybean products, from which estimates of the expected metabolizable energy of optimally heated flakes and beans could be computed. The pertinent data, in Calories per gram of dry matter, were as follows: 50% of protein dehulled soybean-oil meal, 2.81; 44% of protein soybean-oil meal, 2.49; degummed soybean oil, 9.27; crude soybean oil, 9.19. From the composition of the soybeans used in this experiment, it was estimated that 100 parts of dry matter from whole soybeans would yield 19.5 parts of crude soybean oil, and 80.5 parts of 44% protein soybean-oil meal which in turn would be equivalent to approximately 75 parts of 50% protein meal and 5.5 parts of soybean hulls. The latter have been shown in our previous work to have essentially zero metabolizable energy value.

The expected value for properly heated extracted soybean flakes was therefore,

TABLE 3

Effect of heat treatment on growth-promoting value and metabolizable energy of soybeans

Heat treatment of soybeans	Growth data, 4 weeks				Metabolizable energy of soybeans	
	Average weight		Gain/gm feed			
	gm		*gm*		*Cal./gm dry matter*	
None (raw soybeans)	325		0.424		2.81	
	312	*318*[1]	0.405	*0.414*	2.73	2.77
10 minutes, 4 pounds	482		0.637		3.54	
	465	*473*	0.610	*0.623*	3.50	3.52
40 minutes, 4 pounds	479		0.621		3.28	
	468	*473*	0.610	*0.615*	3.41	3.34
60 minutes, 4 pounds	471		0.617		3.40	
	455	*463*	0.610	*0.613*	3.33	3.37
120 minutes, 15 pounds	418		0.507		3.02	
	414	*416*	0.551	*0.529*	3.08	3.05

[1] Figures in italics are averages for duplicate lots; individual lot data in left column for each measurement.

approximately 2.81 Cal./gm of dry matter. The maximal value observed, as shown in table 2, was approximately 93% of this value. This difference is probably meaningful, and may reflect a fundamental difference between commercial processing and the laboratory conditions used in the present experiment. The conditions which we employed, using relatively low pressure for an extended time, were chosen in order to simulate as nearly as possible the time and temperature conditions typical of commercial processing. An important difference between our conditions and the commercial heat treatment following solvent extraction is that the first exposure of solvent-laden flakes to moist heat causes rapid steam distillation of the hydrocarbon solvent, and may produce a physical change in flake structure not achieved by the laboratory processing conditions.

The expected value for properly heated soybeans, based on the values for 44% of protein meal and crude oil, was 3.80 Cal./gm of dry matter. The observed maximal values were in the range 88 to 93% of the expected value, a discrepancy sufficiently large to be meaningful.

The metabolizable energy values provide only information on over-all utilization of soybeans without any indication of whether the meal portion or the oil portion was efficiently utilized. From analyses of excreta and diets for fat, according to the methods indicated above, it was possible to compute the apparent absorbability of the soybean oil in the two series of diets. Data summarizing the results obtained are shown in table 4. In the case of the diets based on soybean flakes, in which degummed soybean oil was employed, the apparent absorbability of the oil in the diets containing heated flakes was 95 to 96%. Because no low-fat diet was included in the experiment, it was not possible to make any direct correction for endogenous fat. However, from other experiments using similar diets we can estimate that the level of endogenous fat excretion would be such as to raise the apparent absorbability of the oil by approximately 2%, so that the true absorbability of soybean oil in this experiment would be of the order of 97 to 98%. This agrees well with other experiments in this laboratory on the absorbability of other samples of degummed soybean oil. Because the soybean oil was highly absorbed in the diets based on soybean flakes, the apparent discrepancy between the expected value and the observed value for heated soybean flakes indicates impaired digestion of protein or carbohydrate.

In contrast, the apparent absorbability of the oil supplied by ground soybeans as shown in table 4 was 76 to 80%, and the true absorbability was probably of the order of 78 to 82%. The absorbability of the oil of heated soybeans was sufficiently

TABLE 4

Apparent absorbability of soybean oil

Heat treatment of soybean flakes or soybeans	Apparent absorbability of oil			
	Soyflake diets		Soybean diets	
	%		%	
None	90		78	
	85	*88*[1]	66	*72*
10 minutes, 4 pounds	95		76	
	95	*95*	76	*76*
40 minutes, 4 pounds	95		81	
	95	*95*	79	*80*
60 minutes, 4 pounds	96		78	
	96	*96*	77	*77*
120 minutes, 15 pounds	97		81	
	95	*96*	80	*80*

[1] Figures in italics are averages for duplicate lots; individual lot data in left column for each type of diet.

TABLE 5

Comparison of computed value of soybeans with observed metabolizable energy (ME)

Heat treatment of soybeans	Apparent absorbability of oil (a)	ME of oil per gm beans (b)[1]	ME of meal portion per gm beans (c)[2]	ME of beans	
				Computed (b+c)	Observed
	%	*Cal.*	*Cal.*	*Cal./gm*	*Cal./gm*
None	72	1.29	1.17	2.46	2.77
10 min., 4 pounds	76	1.36	1.97	3.33	3.52
40 min., 4 pounds	80	1.43	1.95	3.38	3.34
60 min., 4 pounds	77	1.38	1.88	3.26	3.37
120 min., 15 pounds	80	1.43	1.52	2.95	3.05

[1] Computed as 0.195 gm oil × 9.19 Cal. per gm × absorbability.

[2] Computed as 0.75 gm extracted flakes × observed ME from table 2.

low to account for the discrepancy between the observed value for heated soybeans and that expected from previous knowledge of the value of its components. Summarized in table 5 are calculations of the computed value of soybeans based on apparent absorbability of the oil and the metabolizable energy of the heated extracted flakes (taken from table 2). The agreement between the metabolizable energy of the heated soybeans and that estimated from the absorbability of the oil and the metabolizable energy of the meal portion was fairly good. On the average, the computed value was slightly lower than that observed, partly due to the use of apparent absorbability of the oil uncorrected for endogenous fat. In the case of the unheated beans, the computed value was substantially lower than the observed value, suggesting that the utilization of the proteins and/or carbohydrates of the whole bean is higher than in the raw extracted flake, possibly due to a moderating effect of the oil in the ground bean on the action of the trypsin inhibitor.

Therefore, apparently the metabolizable energy of the heated soybean is equal to the sum of the metabolizable energy values of its parts. The low absorbability of the oil in ground soybeans was unexpected in view of the results of our previous experiments in which soybean oil fed as such has shown very high absorbability.

The results of these studies confirm the previous conclusion of others that moist heat treatment improves digestibility of both raw soybeans and extracted soybean flakes. The data obtained in this experiment showed further that the heat treatment which produces maximal rate and efficiency of chick growth also produces maximal utilization as measured by metabolizable energy value. This finding is consistent with the concept that the growth-retarding effect characteristic of unheated soybeans and soybean flakes is due to their poor digestibility in the chick. However, rate of growth is not precisely correlated with metabolizable energy or digestibility, as shown by comparison of the growth data in tables 2 and 3. They show that heated soybeans were equally as effective in promoting rapid growth as the combination of soybean oil plus heated extracted flakes, regardless of the lower absorbability of the oil supplied by the soybeans.

SUMMARY AND CONCLUSIONS

The effect of graded levels of heat treatment of ground soybeans and extracted dehulled soybean flakes (zero, 10, 40 and 60 minutes at 107°C, and 120 minutes at 120°C) on their growth promoting value and metabolizable energy content for chicks was studied. The degree of heat treatment which produced maximal metabolizable energy value also produced maximal rate and efficiency of growth: 10 to 60 minutes at 107°C for extracted flakes, and 10 minutes at 107°C for ground soybeans.

Metabolizable energy content of soybean flakes was somewhat less than that expected from previous study of commercially-processed soybean-oil meals. A possible reason for this discrepancy was discussed.

A large discrepancy between expected and observed values for heated, ground soybeans was obtained. This was found to be due to lower absorbability of soybean oil fed in the form of ground soybeans than soybean oil fed as such. The observed metabolizable energy values for heated soybeans were consistent with the low absorbability of the oil and the observed values of extracted soybean flakes.

The results obtained are consistent with the concept that the low nutritive value of raw soybeans and extracted soybean flakes for the chick is due to their poor digestion and/or absorption.

ACKNOWLEDGMENT

This study was supported in part by a research grant of the National Soybean Processors Association, Chicago, whose assistance is gratefully acknowledged.

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Survival Time and Biochemical Changes in Chicks Fed Diets Lacking Different Essential Amino Acids[1,2]

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An acute deficiency of an essential amino acid in the diet of a growing animal results in an almost immediate cessation of growth and loss of weight. This effect is very different from that of a missing vitamin or mineral, a deficiency of which may not affect growth for some time and normally exhibits characteristic symptoms for different deficiencies.

In investigations of amino acid deficiencies, Rose ('38) found that the lack of valine had the most severe effect on the growth of rats and Almquist and Grau ('44) observed this outcome in chicks following a lack of either isoleucine or valine. However, Almquist ('47) grouped the meager data then existing and postulated a common rate of weight loss for diets lacking any essential amino acid or all protein. He attributed the common figure to the belief that "All the curves converge toward a common negative rate of gain at complete deficiencies of any indispensable amino acid, since protein synthesis must stop in all such cases."

The costs and limited supplies of pure amino acids prevented early workers from carrying chicks for periods long enough to determine the validity of Almquist's theory and from determining how long chicks could survive when fed diets lacking one or more essential amino acids. Even more important, however, was to discover whether significant differences exist between the survival times of chicks fed diets lacking different essential amino acids. In addition, the possibility that animals fed diets lacking particular essential amino acids would exhibit specific syndromes had intrigued early workers, but, unfortunately, the facts failed to support the idea. As a consequence, almost no work has been done on the comparative effects of complete deficiencies of different essential amino acids. These effects could include variations in the rate of weight loss, changes in the body composition and alterations of metabolic patterns.

EXPERIMENTAL METHODS

The effect of diets lacking different essential amino acids on variations in survival time of chicks was investigated. This was coupled with observations on other possible effects of the diets, including free amino acid and peptide levels in the muscle tissue, changes in blood composition, occurrence of lesions and ability of a chick retarded by an amino acid-deficient diet to regain normal growth.

To allow the use of diets devoid of selected amino acids, diets used in these studies consisted of crystalline amino acids, glucose,[4] crude soybean oil, cellulose,[5] vitamins and minerals. Composition of the diets is given in table 1. The amino acid or acids eliminated in each experiment were replaced by glutamic acid except in the amino acid-free diet where they were replaced by glucose. The complete diet normally allowed gains of 3 to 4% per day.

Chicks used in all experiments were Single Comb White Leghorns of the Uni-

Received for publication June 8, 1959.

[1] The material in this paper has been taken from a thesis submitted by the author to the Graduate Division of the University of California in partial satisfaction of the requirements for the degree of Doctor of Philosophy.

[2] A preliminary report of this work is published in Federation Proc., *18:* 540, 1959.

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[4] Cerelose, Corn Products Refining Co., New York.

[5] Solka Floc, Brown Company, Berlin, N. H.

TABLE 1
Composition of diets

	%	%
Amino acid mixture containing		22
DL-Alanine	2.0	
DL-Aspartic acid	1.0	
L-Arginine·HCl	1.4	
L-Cystine	0.5	
L-Glutamic acid	6.0	
Glycine	1.4	
L-Histidine·HCl	0.5	
L-Isoleucine with D-alloisoleucine	1.2	
L-Leucine	1.4	
L-Lysine·HCl	1.4	
DL-Methionine	0.5	
DL-Phenylalanine	0.8	
DL-Serine	0.2	
DL-Threonine	1.0	
DL-Tryptophan	0.4	
L-Tyrosine	0.8	
DL-Valine	1.5	
Crude soybean oil (containing added vitamins A, D, and E)		5
Cellulose		5
Vitamin (except A, D and E) and mineral mixtures (in glucose carrier)[1]		15
Glucose		53
Total		100

[1] Vitamin and mineral levels in the diets were (in mg per 100 gm of diet): thiamine·HCl, 1; riboflavin, 1; pyridoxine·HCl, 1; niacin, 6; *d*-calcium pantothenate, 3; folic acid, 1; biotin, 0.01; menadione, 1; vitamin B_{12} concentrate (1 mg/gm in mannitol), 2.2; choline chloride (25% concentrate), 200; α-tocopherol acetate, 3; vitamin A acetate (1×10^6 units/gm), 1; crystalline vitamin D_3 (4×10^7 I.U./gm), 0.004; diphenyl-para-phenylene-diamine (DPPD), 20; NaCl (iodized), 500; $CaCO_3$, 1920; $CaHPO_4$, 3300; KCl, 600; $MgSO_4 \cdot 7H_2O$, 600; $Na_3SiO_4 \cdot 9H_2O$, 200; $Al_2(SO_4)_3 \cdot 18H_2O$, 100; $MnSO_4 \cdot H_2O$, 30; $ZnSO_4 \cdot 7H_2O$, 6.3; $CuSO_4$, 5; ferric citrate, 74; cobaltous acetate·$4H_2O$; ammonium molybdate·$4H_2O$, 1.3.

versity production strain. They were fed a commercial-type chick starter mash for 10 days after hatching while kept in electrically-heated chick batteries. Chicks were then moved to a room kept at 27 to 30°C where they were housed in small, wire-bottom cages, 4 per cage. Twelve to 16 chicks were fed each diet. Selection at time of transfer was based on uniform size. The very large and very small chicks were discarded. Two males and two females were randomly selected for each cage. After 4 more days on the chick-starter mash, they were weighed and fed the experimental diets. Feed and water were supplied ad libitum with 12 hours of continuous light being given daily. Chicks were weighed daily the first few days and then three times weekly. Observations were made periodically for possible lesions, especially eye lesions and folded tongue (Grau, '45).

Two birds, one male and one female from each lot were killed after 14 days on the experimental diets. These chicks were as near average weight for the lot as possible and were exsanguinated by cardiac puncture. Packed, red-blood-cell volumes were determined by micromethod using 150-mm heparinized capillary tubes. The livers, breast muscle (pectoralis major) and muscles of a thigh and leg were removed, weighed and frozen for later analyses.

As soon as the median survival time of a lot had passed (namely, when the first chick over 50% of the chicks in a lot had died) two of the remaining chicks, as near average weight and condition as those still alive, were fed a commercial-type chick starter to determine whether recovery of normal growth was possible. These chicks were kept until they weighed 250 gm to establish a recovery rate of gain.

The effect of the different diets on the free amino acid levels in de-proteinized muscle extracts was studied by quantitative paper chromatography of extracts prepared from the dissected muscles. Muscle extracts were used instead of blood serum because the free amino acid levels in blood serum were known to be rapidly affected by the level of dietary amino acids; the levels in the muscle were thought to be somewhat more stable. Extracts were prepared, deproteinized and the picric acid removed by the method of Tallan, Moore and Stein ('54). The extract was then freeze-dried, dissolved in several drops of water and transferred to a container where it was made to about 0.1 ml per gm of original muscle and stored in a freezer before chromatography. Most muscle samples weighed 3 to 4 gm.

To investigate the behavior of the muscle stores of carnosine and anserine, muscle extracts were prepared from chicks fed histidine-free diets by the method described above except that instead of being freeze-dried, the extracts were evaporated under vacuum to a point where, after transfer, they were made to 0.5 ml per ml of origi-

nal picric acid supernatant used. This resulted in each milliliter of extract being equivalent to 0.186 gm of muscle. One milliliter of extract was analyzed chromatography (Davey, '57) on 0.9 by 75 cm columns of Dowex 50 by 4 resin in the sodium cycle, using a borate buffer of pH 8, 0.2 N in sodium ions, and containing 0.03% of sodium diethyldithiocarbamate and a detergent (2 ml of a solution of 25 gm of polyoxyethylene lauryl alcohol,[6] in 100 ml of H_2O per 100 ml of buffer). Approximately one milliliter fractions were collected on an automatic fraction collector and analyzed for alpha amino groups with ninhydrin by the method of Rosen ('57). Histidine and carnosine peaks were identified with the aid of a spot test based on the diazo reaction (Feigl, '54). The optical density of the developed fractions was measured on a Model B Beckman spectrophotometer and compared against standards, corrected for dilution and calculated as milligrams per gram of fresh muscle.

[6] Brij-35, supplied by the Atlas Powder Company, Wilmington, Del.

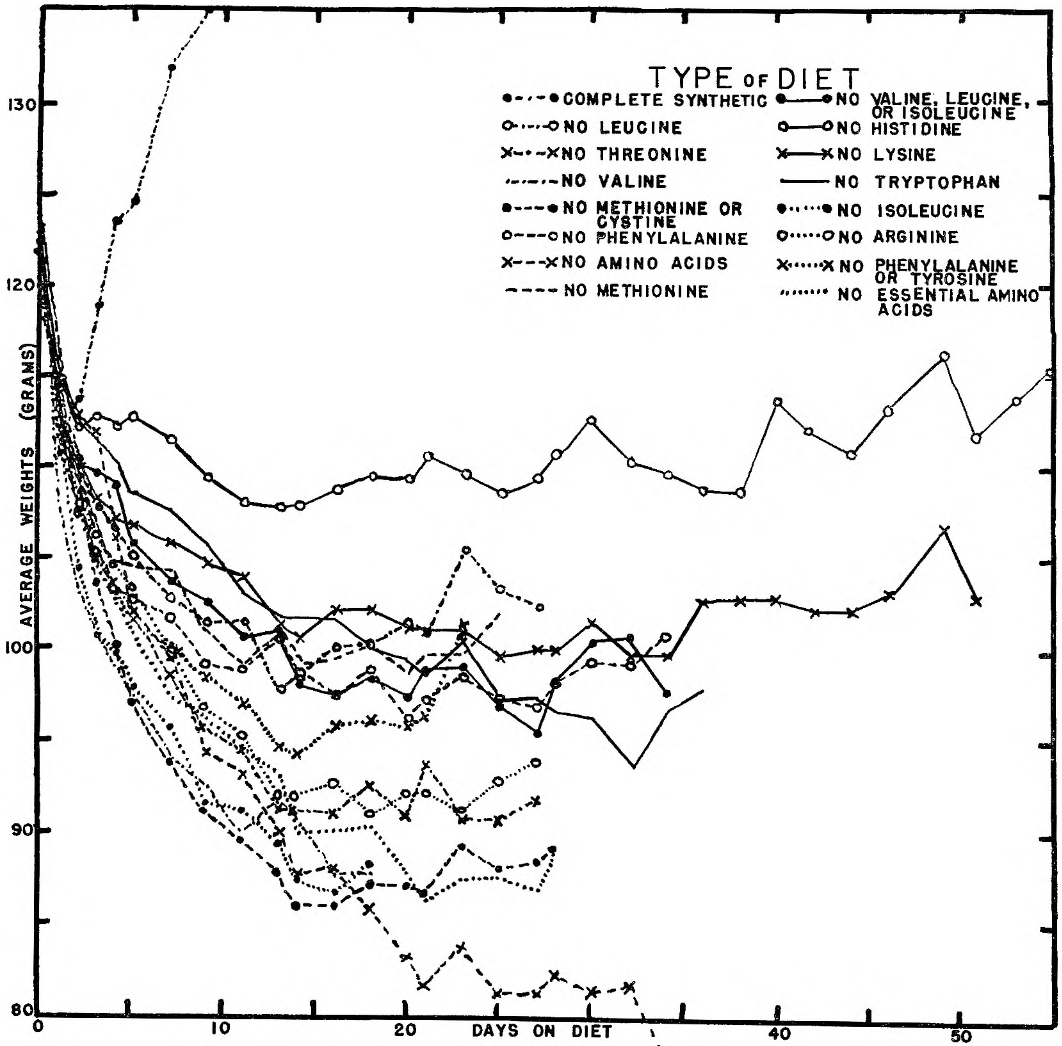

Fig. 1 The average weight of the chicks fed diets lacking different essential amino acids. Curves terminate at the median death time for each lot.

Two chicks were killed each week for 5 weeks for these determinations.

RESULTS

Young chicks fed purified diets based on crystalline amino acid mixtures from which one essential amino acid was omitted showed a characteristic weight curve. They lost weight rapidly for a few days, then remained at a relatively constant weight for variable periods, and finally passed through a short period of rapid weight loss ending in death. The weight lost during the initial period varied considerably; less weight was generally lost with diets which allowed longer survival than with those which resulted in earlier death. The period of stable weight varied widely, depending upon which amino acid was missing from the diet. This indicated either a varying degree of essentiality for the different essential amino acids or the occurrence of factors which modified the effects caused by their absence. Lack of any of the 10 amino acids classed as essential for the chick will result in its death if the chick is fed the diet long enough. During the period of stable weight, the chicks gained in skeletal size, although not at a normal rate, and had essentially normal feather development, apparently at the expense of other body proteins, particularly muscle tissue. Weight curves are given in figure 1. Median survival time for the chicks in each lot, the number observed with folded tongue and the recovery rate of gain for the chicks re-fed a normal diet are shown in table 2. No eye lesions were observed. The low recovery rate of gain of the chicks fed the threonine-free diet was not fully substantiated in later trials.

A direct relationship was noted between the amount of feed consumed and the change in weight for the chicks fed all the diets except the amino acid-free diet (fig. 2). Chicks fed the amino acid-free diet ate much more feed than those fed the essential amino acid-deficient diets while losing

TABLE 2

The median survival times for young chicks fed purified diets lacking various essential amino acids, the lesions observed and recovery rates when re-fed a complete ration

Missing amino acid(s)	Number of chicks	Median survival time	Standard deviation of median	Number with folded tongue	Av. gain/ day after return to normal diet
		days			*gm*
Isoleucine	14	18	3.1	6	14
Valine	14	19	6.1	0	18
Phenylalanine and tyrosine	14	23	6.8	8	12
Methionine	12	22.5	13.7	1	15
Arginine	14	27	5.8	0	11
Threonine	14	27	4.5	2	3[1]
Leucine	14	27.5	9.0	2	16
Methionine and cystine	14	28	6.6	0	14[2]
All essential A.A.	14	28	4.3	2	13
Phenylalanine	14	33.5	12.3	2	13
Valine, leucine and isoleucine	14	34	13.1	7	13
All A.A.	10	35	9.3	1	11
Tryptophan	14	36	4.9	2	12
Lysine	14	52.5	13.0	2	13
Histidine	14	54[3]	26.9[3]	1	11
		60.5[3]	15.7[3]		

[1] One died before reaching 250 gm, the other died one day after reaching 250 gm.

[2] One died before reaching 250 gm, the other was normal.

[3] The first 4 deaths were caused by cannibalism during the first week; if data from these are eliminated, the lower row of figures results. A 60-day survival for chicks fed this diet was substantiated in a later experiment.

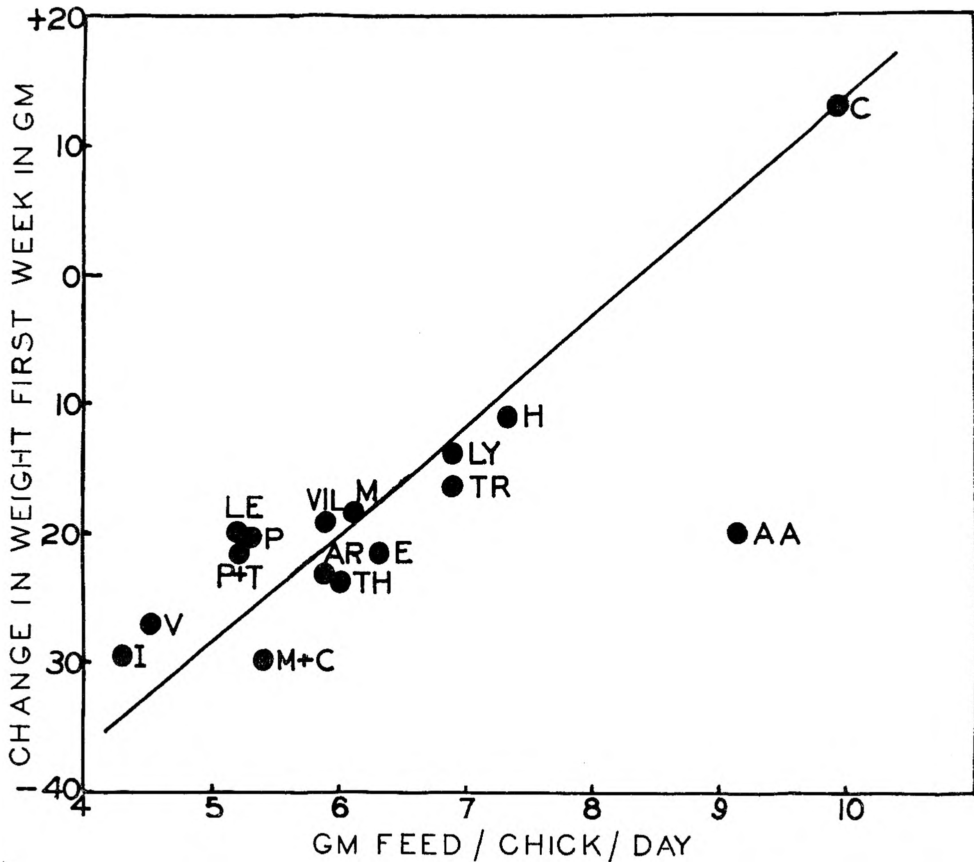

Fig. 2 The relationships between change in weight and feed consumed by chicks fed diets lacking different essential amino acids (first week).

AA, Diet lacking all amino acids
E, Diet lacking all essential amino acids
H, Diet lacking histidine
M, Diet lacking methionine
M+C, Diet lacking methionine and cystine
P, Diet lacking phenylalanine
P+T, Diet lacking phenylalanine and tyrosine
VIL, Diet lacking valine, isoleucine and leucine
AR, Diet lacking arginine
C, Complete diet
I, Diet lacking isoleucine
LE, Diet lacking leucine
LY, Diet lacking lysine
TH, Diet lacking threonine
TR, Diet lacking tryptophan
V, Diet lacking valine

weight at about the same rate. However, they maintained their stores of depot fat longer than the chicks fed the essential amino acid-deficient diets. After two weeks, chicks fed the essential amino acid-deficient diets had lost all visible abdominal fat in contrast to those fed the amino acid-free diet.

In the study of possible reasons for the large differences in the median survival times of the chicks fed the various diets, the free amino acid levels in the muscle tissues from the chicks killed after 14 days on the diets were checked by quantitative paper chromatography. No differences were noticed in the levels of alanine, arginine, phenylalanine or tyrosine which could be ascribed to their lack in the diet. However, the free valine found in the chicks fed the valine-free diet and the free isoleucine in the chicks fed the isoleucine-free diet showed rapid drops, whereas concomitantly the free leucine levels in the chicks fed both diets increased. It is very probable that this tissue free amino acid imbalance contributes to the extreme discomfort exhibited by these chicks and to their early death.

TABLE 3

Changes in the anserine, carnosine and non-protein histidine levels in extracts from the leg and breast muscles of chicks fed histidine-free diets for 5 weeks
Data are given in milligrams per gram of fresh muscle

Weeks on diet	Leg muscle			Breast muscle		
	Histidine	Carnosine	Anserine	Histidine	Carnosine	Anserine
	mg/gm	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*	*mg/gm*
(Initial)	0.06	0.42	1.30	0.07	0.93	5.03
1	0.03	0.32	2.34	0.02	0.33	5.65
2	0.04	0.34	1.75	trace	0.12	6.70
3	0.02	0.37	1.80	0.02	0.08	6.50
4	0.01	0.11	1.55	0.02	0.07	6.50
5	trace	trace	1.88	trace	0.04	7.47

Possible factors which allowed the chicks fed the lysine-free and histidine-free diets to survive longer than the chicks fed the other amino acid-deficient diets were studied. Also, chicks fed these two diets were stronger, more active and lost less weight than those fed the other diets. Observations concerning stores of muscle carnosine (β-alanylhistidine), anserine (β-alanylmethylhistidine) and histidine in chicks fed a histidine-free diet are shown in table 3. The free-histidine level dropped first, followed over a period of weeks by the carnosine level. The anserine level showed an apparent rise; however, since the total amount of muscle was shrinking during this period, the total amount of anserine was quite constant. Apparently carnosine can be removed from the chick's muscles, hydrolyzed to its constituent amino acids and the histidine then used for protein synthesis. Du Vigneaud ('37) observed that dietary carnosine is able to replace histidine for the rat; however, the chick, like the rat[7] (Sakami and Wilson, '44), is unable apparently to demethylate methylhistidine and thus cannot use either that or anserine to replace histidine.

In addition, since Nasset and Gatewood ('54) have shown that the hemoglobin level in the blood falls in rats fed a low-histidine diet, the packed, red-blood-cell volume was determined for a number of chicks fed different diets at various times. Only in the chicks fed histidine-free or lysine-free diets did the red-blood-cell volumes decrease even after the median death times for the lots. The chicks surviving on the histidine-free diet after the median death time, had packed red-blood-cell volumes less than 17% compared with values of 25 to 30% for normal chicks or those fed the other amino acid-deficient diets. Chicks fed the lysine-free diet showed irregular decreases in packed, red-blood-cell volumes.

DISCUSSION

When the diet fed to young chicks lacked one or more of the essential amino acids, the chicks lost weight and died. However, depending upon which amino acids were missing, the weight lost and time before death varied widely. The chick fed this type of diet can synthesize protein only if it can re-use amino acids obtained from the catabolism of proteins or peptides already present in the chick's body, since protein synthesis can occur only when all amino acids needed are present simultaneously (Geiger, '47). When body sources of the missing essential amino acid are exhausted, the chick will die.

The survival time of the experimental chicks appears to fall into three groups: (1) those fed diets lacking either isoleucine or valine, less than 20 days; (2) those fed diets lacking either lysine or histidine, 50 to 60 days; and (3) those fed the other diets, from 23 to 36 days. No evidence was found that the chick could synthesize any amount of an amino acid indispensable for life itself.

Diets lacking isoleucine or valine. The early deaths which occurred when diets lacking either isoleucine or valine were fed suggest that this was a result of the severe free amino acid imbalance which

[7] R. W. Cowgill, and B. Freeberg 1956 Metabolism of methyl-histidine compounds in animals. Federation Proc., *15:* 237 (abstract).

resulted in body tissues when one of these two amino acids was missing. Chicks fed either of these diets exhibited signs of extreme physical distress similar to that caused by some toxic substances. Neither early death nor a large change in the balance of these amino acids in the tissues resulted when both were missing. The free amino acid analyses of the muscle tissue showed that the lack of either of these two amino acids created a greater deficiency of the free amino acid in the tissue than the lack of any other essential amino acid. It is probable that this imbalance (see Deshpande et al., '58a and '58b) of the free amino acids limits the synthesis of necessary protein, and that the lack of these proteins causes the death of the animal.

Diets lacking lysine or histidine. The chicks fed diets lacking either histidine or lysine lost less weight, lived longer and were stronger and more vigorous than chicks fed any of the other deficient diets. This observation led to the investigation of a possible body store of these amino acids. Amino acid storage has never been demonstrated but it is possible that some peptides or proteins could act as emergency reserves of amino acids. Hemoglobin contains relatively large amounts of both lysine and histidine; Block and Weiss ('56) give the lysine content of hemoglobin as 9.1% and the histidine content as 8.0% in contrast to a lysine content of muscle protein of 5.9% and a histidine content of 1.6%. The gradual lowering of the packed, red-blood-cell volumes of the chicks fed the histidine-free diet indicated that the chick may be drawing on the histidine contained in the hemoglobin. Packed, red-blood-cell volumes of the other diets studied were very close to normal even after median death times for their lots.

The large amount of carnosine normally present in the muscle tissue, particularly in the white (breast) muscle, disappeared quite rapidly in chicks fed a histidine-free diet. This suggested that the carnosine was hydrolyzed and the histidine used for protein synthesis. The anserine content of the muscles did not decrease under these conditions. Whether the chick lacks the enzymes necessary to hydrolyze anserine or whether the block is the inability to remove the methyl group from the methylhistidine which would be produced by the hydrolysis was not determined. In view of the previously mentioned inability of a number of animals to use methylhistidine as a dietary replacement for histidine, the latter is probably more important.

Almost normal feather growth and considerable skeletal growth continued in the deficient chicks and thus there appeared to be a high priority for the amino acids necessary for feather and bone formation. Supplies of the amino acid missing in the diet required for feather formation would have had to come from body tissues. Hemoglobin has 27 times the histidine content of feather keratin (Block and Weiss, '56) and 9 times the lysine content. If the chick could utilize the amino acids acquired from the breakdown of relatively small amounts of hemoglobin to form larger amounts of feather keratin, its life could be prolonged. While the relative histidine and lysine content of hemoglobin and bone protein were not as favorable as those of hemoglobin and feather protein, hemoglobin contained 9 times the histidine and twice the lysine content of bone protein. If the amino acid losses in the breakdown of the hemoglobin were not excessive, considerable bone formation could also occur before death takes place.

It is realized that synthesis of proteins other than those of feather and bone must take place. Enzymes, hormones and some cellular replacement continually require certain amounts of the essential amino acids. This demand for amino acids which are not recoverable to a great extent, contributes to the chick's eventual death, even though survival may be prolonged for a time by the use of histidine or lysine from body peptides or proteins for more necessary uses.

Other diets. The survival time of chicks fed diets deficient in essential amino acid did not differ significantly from that of chicks fed diets containing no amino acids with the exception of those lacking either valine, isoleucine, histidine or lysine, which have been discussed. Possibly, the differences could be significant for several of the other diets, but, in general, survival times

were similar. With these diets, however, the main source of amino acids for the necessary protein synthesis is apparently the muscle tissue. Weights of muscles gradually decreased during the experiment; the chicks at death were extremely emaciated. Since feather growth was continued at a near normal rate during this time and there was also considerable skeletal growth, the main movement of amino acids is apparently from muscle tissue to feather and bone formation. Chicks in this group demonstrated a progressive loss of strength and often lost the ability to stand without aid some time before death.

Although the survival time of chicks in this group was similar to that of the chicks receiving no amino acids, many of the effects on the chick were different. Initially, the chicks with no dietary amino acids in the diet appeared to eat to satisfy their energy needs, rather than draw upon stores of body fat as the chicks on essential amino acid-deficient diets. During the first week on an amino acid-free diet they ate considerably more feed than those fed the other diets (fig. 2), but their feed consumption fell rapidly to a level below that of the other chicks. Their activity followed the same pattern as their feed consumption; initially active, they became lethargic as time progressed, and finally, comatose before death. They did not show the rapid disappearance of body fat of the essential amino acid-deficient chicks, but appeared to have almost as rapid feather growth. The metabolic problem in chicks fed amino acid-free diets is quite different from that in the chicks fed a good amount of amino acids which are not usable for protein synthesis. The requirement that high levels of amino acids be deaminated in large quantity before the chick can acquire energy from an essential amino acid deficient diet places an initial demand on the body's fat stores greater than the demands on the chicks fed the amino acid-free diet. It is possible that the ability to metabolize large quantities of amino acids for energy must be developed and when developed, the stable weight period observed in these experiments begins. However, the chicks fed the amino acid-free diet never reached a stable weight (fig. 1); their weight decreased steadily until death.

SUMMARY

Preceding death, three stages were observed in young chicks fed purified amino acid diets, lacking one or more essential amino acids: (1) a rapid loss of weight; (2) a relatively long period of essentially stable weight; and (3) another period of rapid weight loss, followed by death. The loss of weight in the first period and the length of the second period varied with the missing amino acid. Median survival times in days for chicks fed diets lacking the following amino acids are: isoleucine, 18; valine, 19; both phenylalanine and tryrosine, 23; methionine, 26; arginine, 27; threonine, 27; leucine, 28; both methionine and cystine, 28; all essential amino acids, 28; phenylalanine, 34; the combination of valine, isoleucine and leucine, 34; all amino acids, 35; tryptophan, 36; lysine, 53; and histidine, 60. Explanations for these survival times can be categorized as follows: (1) chicks fed diets lacking either isoleucine or valine suffered a severe amino acid imbalance, rapidly affecting the free amino acid levels in tissues and causing early death; (2) chicks fed diets lacking either lysine or histidine survived longer, possibly because of drawing on body stores of peptides (carnosine) and proteins (hemoglobin) especially rich in these amino acids for synthesis of necessary proteins; and (3) chicks fed diets lacking the other essential amino acids, either singly or in combination, survived approximately the same length of time as chicks fed an amino acid-free diet, although the effect on body composition differed. Chicks fed the essential amino acid-deficient diets lost body fat much faster than those fed the amino acid-free diet.

All chicks had near-normal feather development and considerable skeletal growth at the expense of muscle tissue. Except for some chicks fed threonine-free diets, all chicks surviving past the median death time which were re-fed normal, complete diets had a rapid weight gain. The only lesion observed was a varied incidence of folded tongue.

ACKNOWLEDGMENTS

The author wishes to thank Dr. C. R. Grau for his advice and assistance in this

study. Gifts of amino acids by the Dow Chemical Company, Monsanto Chemical Company, E. I. duPont de Nemours, Inc. and International Minerals and Chemical Corporation aided materially in this work.

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The Mineral Requirements of the Dog[1]

I. PHOSPHORUS REQUIREMENT AND AVAILABILITY

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The formulation of a low-phosphorus diet from commercially available feedstuffs to determine the dietary needs of experimental animals is difficult. Kreiger et al. ('41) employed a highly purified ration and reported that the phosphorus of yeast nucleic acid and soybean phospholipids was as readily available as inorganic phosphorus for calcification of bone in the rat. In general, the phosphorus contained in these substances, as well as in ester phosphates and phosphoproteins, compounds which are invariably present when natural feeds are employed, has been assumed to be available.

The objective of the study reported here was (1) to devise a suitable low-phosphorus ration for the dog; (2) to investigate the availability of certain phosphorus-containing compounds found in natural feeds; and (3) to extend knowledge of the minimum phosphorus requirement of the growing dog. Criteria used to test the state of phosphorus nutrition include rate of gain, bone ash, calcium and phosphorus balance data and terminal blood plasma phosphate concentrations.

EXPERIMENTAL

The low-phosphorus rations used in these studies were modifications of those reported by Elmslie and coworkers ('55), altered to provide the nutrient requirements of the growing dog as set forth by the National Research Council ('53) (table 1). The basal ration used in the first experiment was found by analyses of dry matter to contain 0.23% of phosphorus as nucleic acid, phytin, ester-type, phospholipid and inorganic phosphorus (table 2) and 0.13% of calcium before supplementation with calcium carbonate. Certain ingredients in this ration were replaced with low phosphorus products to lower the phosphorus content to 0.17% (0.16% of calcium) for the second experiment. By the addition of anhydrous disodium phosphate, the phosphorus level was increased to 0.33 and 0.53% for the second and third lots of experiment 1. Five levels of phosphorus were used in the second study: 0.17, 0.23, 0.33, 0.43 and 0.53%. The basal rations for both experiments were adjusted to contain 0.61% of calcium.

For the first experiment, a litter of 10 German Shepherd pups was placed according to weight into groups of three, three and 4 dogs. Two litters of German Shepherd and two litters of Shepherd pups provided 4 animals in each lot in experiment 2. The pups were started on experiment when 6 weeks old and fed the respective diets for 10 weeks. Food and water were supplied ad libitum. Vitamin D was provided[3] at the rate of 10 to 15 I.U. per pound of body weight per day. Animals were weighed twice weekly.

They were kept in indoor cages which had expanded metal floors. Precautions were taken against parasites, distemper and canine infectious hepatitis.

Analytical methods. The procedures used for the determination of total phosphorus, acid-soluble phosphorus, phytin phosphorus, inorganic and phospholipid phosphorus were those of Pons et al. ('53).

Received for publication August 3, 1959.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Pet Food Division of the American Feed Manufacturers Association, Chicago.

[2] Present address: Department of Nutrition, Ontario Agricultural College, Guelph, Ontario, Canada.

[3] Haliver Oil.

TABLE 1

Percentage composition of low-phosphorus basal rations[1]

Ingredient	Experiment 1	Experiment 2
	Ration 1	*Ration 2*
Dextrose[2]	36.5	36.7
Yellow corn, ground	30.0	—
Corn/wheat flakes (50/50)[3]	—	25.0
Soybean oil meal	10.0	8.0
Blood meal	10.0	—
Soluble blood[3]	—	8.0
Corn gluten meal	—	4.0
Beet pulp, dried	—	5.0
Alfalfa leaf meal, dehydrated	5.0	5.0
Gelatin	3.0	3.0
Corn oil	2.0	2.0
NaCl (iodized)	1.1	1.0
DL-Methionine	0.2	0.2
Mineral supplement[4]	2.15	2.09
Vitamin supplement[4]	0.083	0.083

[1] Ration 1 contained 19.3% protein and 4.0% fat; ration 2, 18.0% protein and 3.1% fat.

[2] Cerelose.

[3] Low phosphorus products: Flakes, Allied Mills, Inc.; Spray-dried soluble blood, Wilson and Co.

[4] Mineral and vitamin supplements (ration 2 within parentheses): $CaCO_3$, 1.15% (1.09%); KCl, 1.0% (1.0%), with the following added to 100 pounds of the preceding mixture: 0.354 gm $CuSO_4 \cdot 5H_2O$ (0.177 gm); 0.545 gm $CoCl_2 \cdot 6H_2O$ (0.545 gm); 0.236 gm $ZnCl_2$ (0.236 gm); 0.014 gm thiamine (zero); 0.064 gm riboflavin (0.009 gm); 0.236 gm Ca pantothenate (zero); 1.362 gm vitamin B_{12} + carrier, equivalent to 1.362 mg vitamin B_{12} (1.362 mg); 0.136 gm pyridoxine·HCl (0.045 gm); and 36.0 gm choline chloride (36.0 gm). Ration 2 was supplemented also with 0.045 gm niacin.

The crystalline vitamins were kindly provided by Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

Calcium in food, feces and ingesta were determined by the method of Corley and Denis ('25) and plasma calcium by the procedure of Kramer and Tisdall, using the Clark and Collip ('25) modification. Plasma inorganic phosphorus was determined on a trichloracetic acid (TCA) filtrate by the isobutyl alcohol method of Pons and coworkers, ('53). The procedure for chromic oxide was that of Schurch et al. ('50).

When the animals were killed, a femur was removed from each for determination of bone ash. The bone was pulverized, dried, and fat extracted with ethyl ether. After drying to constant weight, the material was ashed at 800 to 1000°F. for 12 hours, cooled in a desiccator and the ash weighed.

RESULTS AND DISCUSSION

The body-weight-gain data (two experiments) are presented in table 3. The animals which received 0.53% of phosphorus gained weight steadily throughout the experiment. Similarly, steady weight gains were observed for the pups given the 0.33% phosphorus level. In contrast, the dogs fed the basal ration (0.23% of phosphorus) grew slowly for the first 5 weeks and little thereafter. These animals showed poor appetite after only 4 to 8 days on experiment and soon became emaciated, inactive and unthrifty. At the end of the experiment, the forelimbs showed extensive bowing and swelling at the wrists.

TABLE 2

Distribution of phosphorus in the basal rations[1]

	Phosphorus			
	Dry matter content		Distribution	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	%	%	%	%
Total phosphorus	0.2300	0.1650	100.0	100.C
Inorganic phosphorus	0.0850	0.0365	37.0	22.1
Phytin phosphorus	0.1080	0.0730	47.0	44.2
Ester-type phosphorus	0.0055	0.0265	2.4	16.1
Nucleic acid phosphorus[2]	0.0250	0.0170	10.8	10.3
Phospholipid phosphorus	0.0065	0.0120	2.8	7.3
Acid-soluble phosphorus	0.1985	0.1360	86.4	82.4

[1] Total P = acid-soluble P + nucleic acid P + phospholipid P. Acid-soluble P = inorganic P + ester-type P + phytin P.

[2] Nucleic acid P and ester-type P were estimated by calculation.

TABLE 3

Summary data of criteria used to indicate the requirement and availability of dietary phosphorus of the weanling dog

	Experiment 1			Experiment 2				
	Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
% Phosphorus	0.23	0.33	0.53	0.17	0.23	0.33	0.43	0.53
Weight gain 70 days	1.15	5.86	6.69	0.72	2.71	7.48	7.52	7.40
Ca absorbed, %	39.2	49.7	58.8	42.3	53.1	65.2	63.6	59.3
P absorbed, %	32.4	52.0	49.2	29.1	63.5	76.1	56.5	44.7
Available P	0.076	0.173	0.370	0.050	0.148	0.256	0.317	0.426
Femur ash (dry-fat free), %	40.8	49.2	51.5	31.5	39.5	51.5	52.0	51.3
Blood plasma P, (mg %)	6.3	7.9	8.5	5.4	7.0	9.0	9.3	8.6

The second experiment was designed to narrow the phosphorus increments for a closer estimate of the requirement of the dog. Inspection of the data of experiment 2 indicates that a dietary level of 0.256% of available phosphorus supported a satisfactory growth rate (fig. 1). The better relative gain for pups receiving 0.33% of phosphorus (experiment 2) was the result of a higher total intake of available phosphorus, since a larger amount of orthophosphate was required to bring the total dietary phosphorus content to 0.33%. The pups fed 0.23% of phosphorus showed a poor, but steady growth response after the first 12 days. At the 0.17% level the body gain was very slight over the 10-week period. Two to three days after the start of the experiment food consumption was greatly reduced, indicating again the rapid effect of phosphorus deprivation upon appetite. The pups showed signs of rickets early in the experiment.

Nine days after the first experiment was started, a complete 12-day collection of feces and urine was made to study the fate of the ingested calcium and phosphorus. The balance data (table 3) indicated that only 52% of the dietary phosphorus at the 0.33% level was absorbed and presumably available (did not appear in the feces). The submarginal growth response of the pups receiving this level demonstrated that 0.173% of available phosphorus in the ration was insufficient. Further evidence for the inadequacy of this level of phosphorus was provided by the slightly lower than normal femur ash

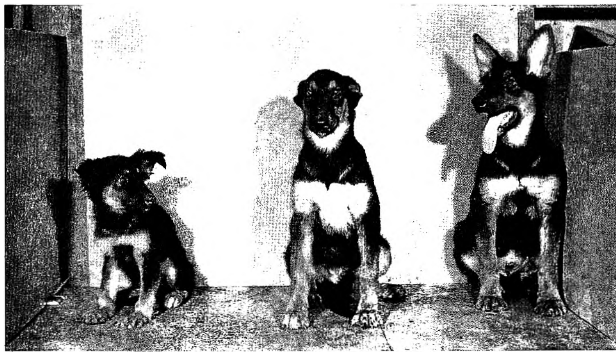

Fig. 1 Effect of phosphorus level on growth of the pup. From left to right, pups fed 0.17, 0.43 and 0.33% of phosphorus, experiment 2.

and plasma inorganic phosphorus values (table 3). The blood-plasma phosphorus concentrations appeared unusually high as recorded in these average figures, but repetition gave similar values. Observations of dogs in our stock colony, when tested at the peak growth period, namely, from 6 to 16 weeks of age, showed from 6.0 to 9.0 mg % of phosphorus, and were thereafter considered to be normal for dogs of this age. (Similar balance data were obtained when the animals had been on experiment for 9 weeks).

In the second experiment, excreta were collected for the 7 days before the end of the experiment. The data (table 3) showed that 76% of the phosphorus was available in ration 2 which contained 0.33% of total phosphorus. Since this was the minimal level which allowed satisfactory growth in this experiment, it indicated that the requirement for available phosphorus was probably not greater than 0.25%.

Maynard ('51) has shown that surplus phosphorus ingested by carnivorus animals was excreted principally in the urine. In these experiments, the low recovery of phosphorus in the urine of the animals in the first two lots of each experiment (table 3) indicated that the requirement was not exceeded at these levels of phosphorus. This suggested incomplete utilization of calcium as a consequence of insufficient phosphorus absorption. The adequacy of the 0.33% phosphorus level was confirmed by the data obtained from femur and blood analyses (table 3) which showed normal bone development and concentration of inorganic phosphorus in the blood plasma.

Calculation of the feed efficiency for the pups in experiment 1 indicated that approximately 15% more food was required by the 0.33%-phosphorus (availability 52%) lot for a gain equivalent to those receiving 0.53%. This evidence indicates that the 0.173% of available phosphorus in the 0.33% ration was below the phosphorus requirement. In the second experiment, however, the pups receiving 0.33, 0.43 or 0.53% phosphorus showed no difference in the amount of feed required to provide a unit gain. Since 77% of the total dietary phosphorus level 0.33% was available (equivalent to 0.25%), this demonstrated that the additional phosphorus supplied by the increment had no effect on rate of gain and that the requirement did not exceed 0.25% total available phosphorus.

An investigation of the availability of dietary phosphorus compounds involves their degradation, absorption and re-excretion in the gastrointestinal tract. Consequently, the phosphorus analyses were made and related to an inert substance (chromic oxide) which was incorporated into the ration two weeks before the first experiment was terminated. When the animals were killed, the contents of the gastrointestinal tract were removed from the stomach, colon and three equally-measured lengths of the small intestine.

The data indicated (table 4) a considerable increase in concentration of inorganic phosphorus in the upper small intestine. The slightly higher value obtained for the pups in experiment 2, given 0.25% of available phosphorus (dietary level, 0.33% of phosphorus) suggested that the requirement was close to this level. Feeding a dietary level of phosphorus greater than 0.33% exceeded the requirement and part of the absorbed phosphorus was excreted in the urine. Evidence was obtained also in experiment 2 where the lower levels of dietary phosphorus resulted in increased amounts of calcium excretion in the urine. The inorganic phosphorus released from the organically bound phosphorus was a minor source at this point and apparently a major amount originated from secretion into the gut. Analyses of bile for total phosphorus concentration gave values of 92 mg% of phosphorus for animals in lot 1, and 104 and 90 mg% of phosphorus for animals in lots 2 and 3, respectively. The amount of inorganic phosphorus in the bile samples was not determined.

The indication here, that inorganic phosphorus was secreted into the lumen of the small intestine of the dog agrees with the observations of Marek et al. ('35) and Moore and Tyler ('55) who reported similar findings for the pig. In addition, evidence has been presented for the secretion of phosphorus into the upper small intestine of the rat (Fournier, '50).

TABLE 4
Calcium and certain phosphorus compounds in feed, feces and ingesta (%)[1]
Experiment 1

	Phosphorus content						
	Feed	Stomach	Small intestine[2]			Colon	Feces[3]
			Upper	Middle	Lower		
Lot 1							
Nucleic acid P	0.021	0.054	0.078	0.034	0.016	0.013	0.013
Phospholipid P	0.005	0.011	0.033	0.005	0.002	0.003	0.004
Inorganic P	0.071	0.078	0.256	0.141	0.097	0.077	0.056
Phytin P	0.091	0.088	0.083	0.078	0.077	0.073	0.074
Ester-type P	0.005	0.016	0.049	0.009	0.009	0.005	0.006
Acid-soluble P	0.167	0.182	0.388	0.228	0.183	0.155	0.136
Total P	0.193	0.247	0.499	0.267	0.201	0.171	0.153
Calcium	0.513	0.509	0.221	0.335	0.278	0.243	0.281
Lot 2							
Nucleic acid P	0.021	0.028	0.020	0.038	0.024	0.014	0.010
Phospholipid P	0.005	0.001	0.024	0.003	0.002	0.005	0.003
Inorganic P	0.155	0.175	0.417	0.201	0.141	0.081	0.057
Phytin P	0.091	0.087	0.078	0.074	0.070	0.071	0.067
Ester-type P	0.005	0.011	0.032	0.021	0.007	0.004	0.000
Acid-soluble P	0.251	0.273	0.527	0.296	0.218	0.156	0.124
Total P	0.277	0.302	0.571	0.337	0.244	0.175	0.137
Calcium	0.513	0.468	0.282	0.457	0.364	0.245	0.266
Lot 3							
Nucleic acid P	0.021	0.027	0.031	0.027	0.014	0.008	0.004
Phospholipid P	0.005	0.006	0.041	0.002	0.001	0.002	0.002
Inorganic P	0.324	0.336	0.492	0.260	0.136	0.089	0.066
Phytin P	0.091	0.085	0.077	0.069	0.066	0.057	0.058
Ester-type P	0.005	0.021	0.046	0.009	0.003	0.004	0.001
Acid-soluble P	0.420	0.442	0.615	0.338	0.205	0.150	0.125
Total P	0.446	0.475	0.687	0.367	0.220	0.160	0.131
Calcium	0.513	0.491	0.250	0.389	0.272	0.225	0.242

[1] Expressed as $\frac{\text{\% phosphorus (or calcium) in dry matter}}{\text{\% chromic oxide in dry matter}}$.

[2] The small intestine was measured into three parts of equal length.

[3] Average value for a 12-day collection of feces.

Inspection of the data indicated also that large quantities of inorganic phosphorus were absorbed in the second section of the small intestine and smaller amounts from the lower small intestine and colon. A considerable absorption of calcium occurred from the contents of the upper section of the small intestine and less from the terminal third. The high concentration of calcium in the second section suggests an excretion of calcium into the tract.

Phospholipid phosphorus, a small portion of the total dietary phosphorus, underwent a 5 to 8-fold increase in concentration in the upper section of the small intestine and was absorbed almost completely in the remainder of the tract. Ester-type phosphorus appeared to react in a similar manner.

Values for nucleic acid phosphorus, obtained by calculation, were variable. The results indicate, however, that nucleic acid breakdown occurred to a large extent in the lower section of the small intestine. Although the availability of phytin phosphorus for animals has been studied, the extent of phytin hydrolysis in various sections of the gastrointestinal tract and the hydrolysis of substances effecting this breakdown are not well known. In the work reported here, the data indicated that the degradation of phytin occurred chiefly in the first two-thirds of the small intestine, although some hydrolysis also took place in other sections of the digestive

tract. The breakdown of phytin in the stomach of the pups presumably occurred in the cardiac section during the earlier stages of digestion and before the pH of the stomach contents became sufficiently low to inhibit phytase activity (Moore and Tyler, '55). Hill and Tyler ('54) have presented data indicating the irreversible inactivation of cereal phytase at pH values of 2 to 3.

The addition of inorganic phosphate to the basal ration improved the utilization of phytin phosphorus, possibly the result of an improved calcium to phosphorus ratio. In this respect, the data revealed that apart from phytin phosphorus, the dietary calcium to phosphorus ratio did not reduce markedly the utilization of other forms of phosphorus studied except perhaps inorganic phosphate.

In the second experiment, the gastrointestinal contents were not analyzed for phosphorus compounds but determinations were made on feed and fecal samples taken for a 7-day period at the end of the experiment. Fecal concentrations of phospholipid and ester-type phosphorus were similar to those obtained in the first study and indicated that 10 to 25% of the quantity ingested was excreted in the feces. The utilization of phytin and inorganic phosphorus at the adequate levels of phosphorus was similar to those observed for the 0.53% lot in experiment 1 but markedly poorer at the 0.17 and 0.23% phosphorus levels (high calcium/phosphorus ratios).

SUMMARY

The phosphorus requirement of the dog has been established with a natural diet which contained 0.33% of phosphorus, of which 76% was shown to be available. Hence, it is believed that the marginal phosphorus requirement corrected for unavailable phosphorus was 0.25%. This confirms previous work.

Data have been obtained which demonstrate the appearance of inorganic, phospholipid, ester-type and nucleic acid phosphorus in the gastrointestinal tract from non-dietary sources. The subsequent decrease in concentration of these compounds in one or more sections of the tract demonstrated that these forms of phosphorus were largely available to the dog.

Twenty to 40% of the ingested phytin was degraded with better utilization of phytin phosphorus at higher levels of inorganic phosphate (Ca/phosphorus ratios nearer to 1.2). The availability of phosphorus other than that from phytin or inorganic phosphate did not appear to be affected by the dietary Ca/phosphorus ratio.

ACKNOWLEDGMENT

We are indebted to Dr. Tadeusz Kowalczyk for his help in the maintenance of the health of the dogs.

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The Mineral Requirements of the Dog[1]

II. THE RELATION OF CALCIUM, PHOSPHORUS AND FAT LEVELS TO MINIMAL CALCIUM AND PHOSPHORUS REQUIREMENTS

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Data obtained from recent investigations (Jenkins and Phillips, '60) indicated that the minimal available phosphorus requirement of the dog for growth and calcification of bone is near 0.25%. The usefulness of a minimal-requirement value is obviously limited by dietary factors which affect the availability of the element. A study was initiated to investigate the quantitative changes in the requirement when the level of one or more of these factors is altered.

The marked influence of calcium on the utilization of phosphorus is recognized, although attempts to establish or dispute the converse have led to conflicting results. Notwithstanding, additional knowledge is needed concerning the interdependence of calcium and phosphorus in the zone of their marginal dietary requirements.

The studies to be reported were designed to provide an estimate of the minimal calcium requirement, and determine the effect of dietary levels of calcium, phosphorus and fat on the minimal required intake of calcium and phosphorus by the growing dog.

EXPERIMENTAL

Four 10-week experiments were conducted using weanling pups. Littermate animals were alloted to provide a similar average-starting-weight and an equal number of dogs for each experimental group. Housing consisted of expanded-metal-bottom cages with 2 to 3 pups per cage. Protection was provided against parasites, distemper and canine infectious hepatitis.

The composition of the low phosphorus basal ration used in the first three experiments has been described previously (Jenkins and Phillips, '60). For the high-fat experiment, the ration was modified to have the following percentage composition: dextrose,[3] 13.2; corn/wheat flakes, 25.0; soybean oil meal, 9.0; soluble blood, 10.0; corn gluten meal, 5.0; dried beet pulp, 5.0; dehydrated alfalfa leaf meal, 5.0; wheat gluten, 6.6; white grease, stabilized,[4] 9.9; NaCl, iodized, 1.0; DL-methionine, 0.2; KCl, 1.0; and to 100 pounds of the preceding, the following was added (in grams): $CuSO_4 \cdot 5H_2O$, 0.177; $CoCl_2 \cdot 6H_2O$, 0.545; $ZnCl_2$, 0.236; riboflavin, 0.018; niacin, 0.136; vitamin B_{12} (in carrier), 1.362; pyridoxine·HCl, 0.045; and choline chloride, 48.0. The ration contained 0.20 P, 0.17 Ca, 24 protein and 20% fat. Changes in the calcium and phosphorus content were brought about by the addition of $CaCO_3$, anhydrous Na_2HPO_4 and $(NH_4)_2HPO_4$, at the expense of dextrose. Various mixtures of the phosphorus salts were used to maintain a constant sodium content in each ration.

Received for publication August 3, 1959.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Pet Food Division of the American Feed Manufacturers Association, Chicago. The crystalline vitamins were kindly provided by Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

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[3] Cerelose; Corn Products Refining Co.

[4] Spurt, (inedible lard) obtained from Oscar Mayer Company, Madison, Wisconsin.

Vitamin D[5] was administered to the animals by dropper every three days at a level of 10 to 15 I.U./pound of body weight per day. Food and water were provided ad libitum.

The concentrations of inorganic, ester-type and phospholipid phosphorus in the low, and high-fat rations were similar. The increase in concentration of phytin and nucleic acid phosphorus accounted for most of the increase in phosphorus content from 0.165 to 0.20%.

The methods used for (1) total phosphorus, phytin phosphorus and calcium in feed and fecal samples; (2) femur ash; and (3) plasma calcium and inorganic phosphorus concentrations, have been described by Jenkins and Phillips ('60). Calcium and phosphorus in urine samples were determined by the procedure of Clark and Collip ('25) and the isobutyl alcohol method of Pons et al. ('53).

The calcium and phosphorus requirements and interdependence. The first experiment was designed to study the effect of calcium intake on the utilization of a marginal level of phosphorus, and to estimate the calcium requirement of the growing dog. Four lots of animals were fed a ration containing 0.33% of phosphorus and either 0.30, 0.60, 0.90 or 1.20% of calcium. Data obtained in this study indicated that phosphorus availability was inhibited by calcium imbalance. A second experiment followed to ascertain whether this effect could be prevented by an appropriate increase in the phosphorus intake. Finally, a third experiment was conducted to investigate the effect of phosphorus intake on the growth response of weanling pups fed a marginal amount of calcium.

RESULTS AND DISCUSSION

The growth data are presented in table 1. In this series of experiments, the lot-2 animals (experiment 1) which received 0.60% of calcium and 0.33% of phosphorus, made excellent body gains throughout the 10-week growth period. The growth response of the lot-1 pups indicated that 0.30% of calcium was slightly less than the requirement. Feeding calcium at the level of 0.90 and 1.20% greatly reduced the rate of growth. In the second experiment, the pups which received 1.20% of calcium and 0.33% likewise made poor gains in body weight, thus verifying the results obtained in the first experiment. When the phosphorus level was raised to 1.00% and the calcium level kept at 1.20%, thus restoring calcium/phosphorus balance at a higher plane of mineral nutrition, the growth rate returned to approximately normal. The weight gains and food consumption data suggested that the lowered gain may have been

[5] Haliver Oil.

TABLE 1

Summary data on the calcium requirements as indicated by weight gain[1]

70-Day period	Experiment 1				Experiment 2			Experiment 3		
	Lot 1	Lot 2[2]	Lot 3	Lot 4	Lot 1[2]	Lot 2	Lot 3	Lot 1[2]	Lot 2	Lot 3
% P	0.33	0.33	0.33	0.33	0.33	0.33	1.00	0.33	0.65	1.00
% Ca	0.30	0.60	0.90	1.20	0.60	1.20	1.20	0.30	0.30	0.30
Weight gain/kg	6.68	7.18	3.78	1.89	4.31	1.28	3.99	5.43	5.38	4.99

	Lot 1[3]	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7
% P	0.33	0.33	0.41	0.41	0.41	0.41	0.20
% Ca	0.30	0.30	0.37	0.47	0.57	0.67	0.17
% Fat	3.0	20.0	20.0	20.0	20.0	20.0	20.0
Weight gain/kg	5.62	7.04	7.48	7.20	7.32	6.94	4.88

[1] Experiment 1. Four litters of Shepherd-Collie dogs; 4 pups per lot.
Experiment 2. One litter Shepherds, one Terriers, two Beagles; 4 pups per lot.
Experiment 3. Four litters Shepherds, one Cocker Spaniels, one Beagles; 6 pups per lot.

[2] Control lot.

[3] Low-fat control.

the consequence of a less palatable ration. In any case, the use of high levels of calcium and phosphorus appeared to have no advantage over the lower intakes for acceptable growth, health and well-being.

The body-weight data obtained in the third experiment demonstrated that the pups receiving a marginal calcium intake grew equally well whether a ration with a phosphorus/calcium ratio of 2.17 or 1.10 was used. An increase in the ratio to 3.33 by the addition of inorganic phosphate reduced slightly the average cumulative gain by 10 to 15%. All the animals in each lot showed good physical appearance and no outward signs of rickets. These data are in good agreement with the findings of Nicholaysen ('37) for rats and Farquharson ('31) for dogs and indicate that excess phosphorus intake had only a slight effect on calcium utilization. It must be recalled, however, that in the experiment of Farquharson, as well as in the study reported here, inorganic phosphate was added to raise the dietary phosphorus level. One might rightly expect a more variable effect of phosphorus on calcium utilization where a natural source of phosphorus was used or a considerable amount of phytate was present (Hoff-Jorgensen, '45).

For the 10 terminal days of the first and second experiment, the excreta were collected quantitatively and analyzed for calcium and phosphorus. The balance data for experiment 1 (table 2) show that the higher calcium intakes reduced the availability and function of phosphorus and increased the amount excreted in the feces. The large percentage of dietary phosphorus recovered in the urine of the animals in lot 1, indicated that the absorbed phosphorus was poorly retained. Because of the low recovery of calcium in the urine of the pups in lot 1, this effect was apparently the result of a marginal intake of calcium. The greatest weight gain was made by the animals in lot 2, experiment 1, pups (table 1). The total available phosphorus was 0.22% and of calcium, 0.37% (table 2). The 0.22% figure obtained in this experiment agrees

TABLE 2

Relation of dietary calcium and phosphorus contents to retention and excretion

Lot no.	Dietary		Calcium distribution (intake)			Phosphorus distribution (intake)			Available P and Ca in ration[1]	
	P	Ca	Excreted Urine	Excreted Feces	Retained	Excreted Urine	Excreted Feces	Retained	P	Ca
	%	%	%	%	%	%	%	%	%	%
					Experiment 1					
1	0.33	0.30	0.6	42.3	57.1	12.8	28.9	58.3	0.235	0.173
2	0.33	0.60	3.6	37.9	58.5	2.1	33.4	64.5	0.220	0.373
3	0.33	0.90	3.9	57.6	38.5	1.1	42.9	56.0	0.188	0.382
4	0.33	1.20	8.3	49.5	42.2	0.6	51.0	48.4	0.162	0.606
					Experiment 2					
1	0.33	0.60	2.5	34.4	63.1	1.6	32.1	66.3	0.220	0.394
2	0.33	1.20	6.8	59.4	33.8	0.4	57.0	42.6	0.142	0.487
3	1.00	1.20	2.0	68.0	30.0	31.9	50.0	18.1	0.500	0.384
					High fat experiment					
1[2]	0.33	0.30	0.1	30.1	69.8	19.0	29.2	51.8	0.234	0.210
2	0.33	0.30	0.1	37.6	62.3	18.5	32.2	49.3	0.224	0.187
3	0.41	0.37	0.1	22.1	77.8	17.5	25.0	57.5	0.308	0.288
4	0.41	0.47	0.2	36.2	63.6	5.0	25.5	69.5	0.305	0.300
5	0.41	0.57	1.2	36.6	62.2	3.8	27.8	68.4	0.296	0.361
6	0.41	0.67	5.2	42.3	62.5	3.6	29.8	66.6	0.288	0.387
7[3]	0.20	0.17	0.6	32.2	67.2	9.2	20.8	70.0	0.158	0.115

[1] Available $P = \frac{\text{dietary P (gm)} - \text{fecal P (gm)}}{\text{dietary dry matter (gm)}} \times (100)$

[2] Low-fat control.

[3] High-fat basal 20%.

reasonably well with the 0.25% value for phosphorus found in previous studies (Jenkins and Phillips, '60 and others). An increase in the dietary level of calcium from 0.60 to 0.90%, with 0.33% of phosphorus, decreased the phosphorus availability by 15%. A further increase in the calcium intake to 1.20% reduced the phosphorus availability by 26%. The poor growth of the high-calcium fed animals was apparently the result of both low food consumption and general inanition, and the metabolic effects of phosphorus deprivation. Nevertheless, the growth rate reflected indirectly the degree of phosphorus deficiency. The femur ash and terminal plasma inorganic phosphorus data, presented in table 3, provide further evidence that high calcium levels inhibit phosphorus utilization. The 0.30% calcium level (01.7% of available calcium), suggest a minimal or submarginal concentration of dietary calcium for the fast growing dog.

The balance data for the second experiment (table 2) demonstrated again the marked effect of calcium intake on phosphorus availability. Elevation of the phosphorus intake from 0.33 to 1.00% in lot 3, experiment 2, improved food consumption and phosphorus retention to values only slightly below those of the control lot. In this experiment, the minimum calcium and phosphorus requirements (lot 2) were found to be 0.39 and 0.22%, respectively. It is of interest that in addition to the lowered food consumption and weight gain shown for the high intakes of calcium and phosphorus, the femur ash value was also slightly lower in each instance than that of the litter-mate controls (lot 1, table 3). The significance of this latter finding is not readily apparent but could have been related to the observed decrease in food consumption.

The relation of dietary fat to the calcium and phosphorus requirements. The aim of this study was to investigate the effect of dietary fat level on the minimum calcium and phosphorus requirements of the growing dog.

Various amounts of calcium carbonate and phosphorus salts were added to the basal ration to provide lots 2 to 6 (see table 2) and a control lot 7. A low-fat (3.0%) control group was included as lot 1. The high-fat diet was found, by calculation, to have an energy concentration 25% higher than the low-fat basal. Consequently, the marginal levels of calcium

TABLE 3

The effect of dietary levels of calcium, phosphorus and fat on femur-ash content and plasma concentration of calcium and inorganic phosphorus

Dietary		Ash in dry, fat-free femur	Plasma[1]	
Ca	P	Average	Calcium	Incrganic
%	%	%	mg %	mg %
		Experiment 1		
0.30	0.33	48.7	8.4	9.1
0.60	0.33	50.1	9.8	8.5
0.90	0.33	43.8	10.6	8.0
1.20	0.33	40.9	10.7	6.8
		Experiment 2		
0.60	0.33	49.7	10.8	8.7
1.20	0.33	39.6	12.1	5.6
1.20	1.00	47.7	8.1	9.5
		High-fat experiment		
0.30	0.33	48.5	8.4	8.8
0.30	0.33	46.9	8.4	8.9
0.37	0.41	47.7	8.2	9.3
0.47	0.41	48.8	9.8	9.1
0.57	0.41	51.3	11.1	8.6
0.67	0.41	52.1	9.9	8.9
0.17	0.20	42.1	8.0	8.8

[1] Terminal plasma values.

and phosphorus for the low-fat ration (lot 1) were increased by 25% (lot 3).

Thus, pups receiving the high-fat basal ration (lot 7) showed good appetite and weight gains despite the very low dietary levels of calcium and phosphorus (table 1). It is of interest that in all previous investigations where phosphorus was the deficient element, the authors have observed a concomitant reduction of appetite and plasma inorganic phosphorus concentration. In this experiment, however, although the basal intakes of calcium and phosphorus were both low, calcium was the limiting deficiency.

Inspection of the data concerning blood revealed that despite a low intake of phosphorus, increased retention of the absorbed element allowed a normal plasma inorganic phosphorus level. It appears then that the maintenance of appetite may have been related to the plasma inorganic phosphorus concentration.

Despite the good appetite shown by the lot 7 dogs, the inadequacy of the calcium and phosphorus intakes became evident early in the experiment. Four weeks after the start of the study, the pups began to show bowing of the forelegs, swelling at the joints and poor gait.

The expected differences in weight gain between the animals in lots 2, 3, 4 and 5 were apparently obscured by fat deposition. The low-fat group (3.0%) (lot 1) showed less favorable body-weight gains than any of the high-fat lots which received additional calcium and phosphorus. However, postmortem examination revealed extensive fat deposition in the high-fat fed pups and this could well have accounted for the greater weight gains.

The balance data (table 2) indicated that for suboptimal intakes of calcium and phosphorus, the pups receiving the low-fat ration retained larger quantities of these elements (lot 1 vs. lot 2). Examination of the results, however, showed that this effect could be acccunted for by the difference in food consumption. When this latter difference was nullified by the use of proportionately higher calcium and phosphorus levels, the amounts retained were comparable (lot 1 vs. lot 3).

TABLE 4

The relation of calcium and phosphorus intakes to phytin phosphorus availability

Character of diet	Dietary level		Ca/P ratio	Ca/phytin P ratio	Phytin digestibility[1]
	Calcium	Phosphorus			
	%	%			%
High fat (20%)	0.17	0.20	0.85	2.42	99.4
High fat (20%)	0.30	0.33	0.91	3.63	85.8
Low fat (3.0%)	0.30	0.33	0.91	4.11	80.4
Ca/P ratio (low Ca, low P)	0.30	0.33	0.91	4.11	76.4
High fat	0.37	0.41	0.90	4.48	84.2
High fat	0.47	0.41	1.15	5.69	72.4
High fat	0.57	0.41	1.39	6.90	64.8
High fat	0.67	0.41	1.63	8.11	.57.0
Ca/P ratio (high Ca, low P)	0.60	0.33	1.82	8.22	59.7
Balance, Ca and P for growth	0.60	0.33	1.82	8.22	62.5
P requirement + safety margin[2]	0.61	0.53	1.15[3]	8.36[3]	60.2
P requirement + safety margin[2]	0.61	0.43	1.42	8.36	60.3
P requirement + safety margin[2]	0.61	0.33	1.85	8.36	63.7
P requirement + safety margin[2]	0.61	0.23	2.65	8.36	56.1
P requirement + safety margin[2]	0.61	0.17	3.59	8.36	51.7
Ca/P ratio (high Ca, low P)	0.90	0.33	2.73	12.33	51.3
Balance, Ca and P	1.20	1.00	1.20[3]	16.44[3]	47.7
Imbalance, Ca and P	1.20	0.33	3.64[3]	16.44[3]	43.1
Ca/P ratio (high Ca, low P)	1.20	0.33	3.64[3]	16.44[3]	34.9

[1] $\text{Phytin P} = \frac{(\text{phytin P in feed, gm} - \text{phytin P in feces, gm})}{(\text{phytin P in feed, gm})} \times (100)$

[2] (Jenkins and Phillips, '60).

[3] Indicates where Ca/phytin P ratio appears to fit better.

The balance, femur and blood data indicated that elevation of the fat content of the ration from 3 to 20%, increased the available phosphorus requirement from 0.22 to 0.30%. The calcium requirement did not appear to be altered appreciably and remained at 0.36 to 0.39%

The relation of calcium and phosphorus intakes to phytin phosphorus availability. During the course of studies on the availability of phytin phosphorus for the growing dog, data have accumulated which indicated that the calcium phytin phosphorus ratio of a ration may be of greater significance than the calcium/total phosphorus ratio. Table 4 summarizes results obtained from balance trials conducted during the last 10 days of the experiments reported in this paper. The data have been listed first accordingly to the dietary calcium/phytin phosphorus ratio and secondly with respect to the calcium/total phosphorus ratio. It may be seen that several of the values stress the importance of the calcium/phytin ratio. Although there was a certain amount of variation, the remaining data demonstrated that the availability of phytin tended to improve at the higher phosphorus intakes. Of particular interest are the results of the second experiment (see "Balance, Ca and P"). These data demonstrated that a high dietary level of calcium markedly interfered with the utilization of phytin phosphorus. The addition of inorganic phosphorus to bring the calcium/phosphorus ratio to a desirable figure, increased the phytin phosphorus utilization to only a small extent. At the lower levels of calcium and phosphorus (with a similar dietary calcium/phosphorus ratio) phytin phosphorus utilization was markedly greater.

SUMMARY

A study has been made of certain mineral requirements of the growing dog and factors which affect them. Data obtained from these studies indicate that an interdependence exists among certain mineral elements which influence the requirements profoundly.

It has been demonstrated that the growing dog required 0.37 of calcium as a minimal level to provide a satisfactory growth rate, normal development and well-mineralized skeletal structures. Doubling or tripling the dietary phosphorus in a ration just adequate in calcium increased the calcium requirement as measured by growth. The requirement was unaffected by a high fat diet.

Diets which contained calcium in excess of 0.60% reduced the availability, function and the retention of phosphorus in growing dogs fed a ration just adequate in phosphorus. In contrast to the negligible effect of high dietary fat (20%) upon the need for calcium, the high-fat diet increased the phosphorus needs of the ration from 0.22 to 0.30%, an increase necessitated by a reduced feed ingestion.

Evidence was obtained which showed that phytin phosphorus was utilized better at low dietary levels of calcium. It was observed that the availability of phytin phosphorus in some instances was more closely related to the dietary calcium/phytin phosphorus ratio than to the ratio of calcium/total phosphorus.

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Effect of Saturated and Unsaturated Fatty Acids on Dietary Lipogranuloma[1]

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Occurrence of lipogranuloma in adipose tissue of rats fed saturated fats has been reported recently by Cox and DeEds ('58), by Herting and Crain ('58) and by Ambrose et al. ('58a). This foreign-body type reaction is characterized by multinucleation and residual eosinophilic material within the fat cell. The photomicrograph shows typical lipogranuloma (fig. 1).

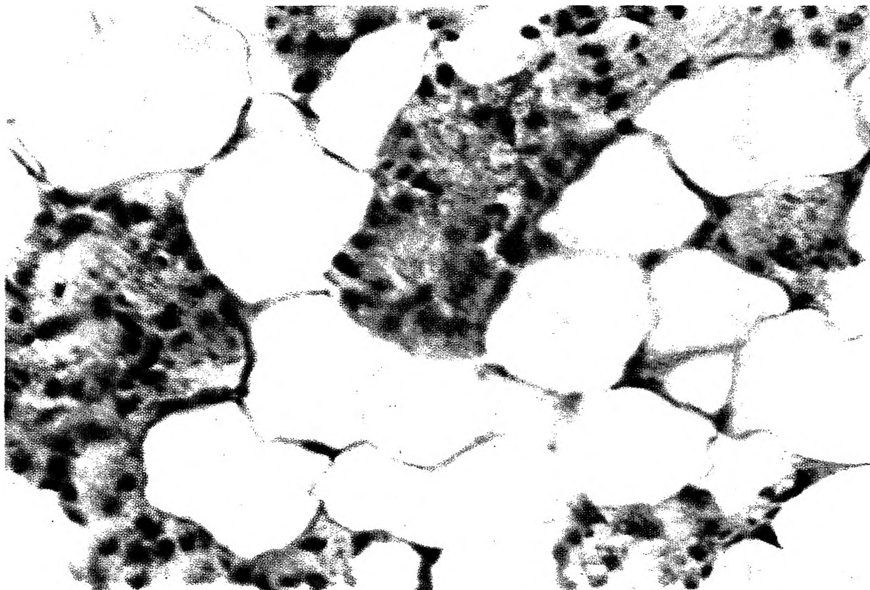

Fig. 1 Typical lipogranuloma. Affected cells (stained) are interspersed among normal cells (clear). Hematoxylin and eosin. × 340.

Easy reversibility of lipogranuloma by dietary means was shown by Herting et al. ('59). Merely changing dietary fat from a saturated to an unsaturated type led to rapid diminution and eventual disappearance of the reaction. This indicated that a sufficient proportion of unsaturated to saturated fat in the diet would prevent development of lipogranuloma. An experiment establishing this critical ratio is described here, concerning which a preliminary report was given by Herting et al.[2]

METHODS

Weanling rats,[3] distributed randomly into groups of 48 animals, equal males and

Received for publication July 6, 1959.

[1] Communication no. 254, Research Laboratories, Distillation Products Industries.

[2] D. C. Herting, P. L. Harris and R. C. Crain 1959 Dietary factors influencing lipogranuloma. Federation Proc., *18:* 529 (abstract).

[3] Holtzman Rat Company.

TABLE 1

Source and proportion of saturated and unsaturated fatty acids in diets used to produce lipogranuloma

Group	Dietary fat[1]		Fatty acid			
			Unsaturated	Saturated		
	Acetylated monoglycerides[2]	Corn oil-lard[3]	Corn oil-lard	Acetylated monoglycerides	Corn oil-lard	Unsat./sat.
	%	%	%	%	%	%
1	50.0	—	—	35.4	—	—
2	40.0	7.4	5.3	28.3	1.8	0.17
3	33.5	12.2	8.7	23.7	2.9	0.33
4	25.0	18.5	13.3	17.7	4.4	0.60
5	16.5	24.8	17.8	11.7	5.9	1.0
6	10.0	29.6	21.2	7.1	7.1	1.5
7	—	37.0	26.5	—	8.8	3.0

[1] Diets also contained in per cent, vitamin-test casein 20, B-vitaminized casein 4, U.S.P. XIV salts 5, fat-soluble vitamins, and glucose to make up the difference between the above ingredients and 100%.

[2] Distilled, partially acetylated monoglycerides made from hydrogenated lard (Herting et al., '55).

[3] One to one mixture of lard and corn oil, calculated to contain 39 and 11%, respectively, of saturated fatty acids.

females in each, were housed in groups of 4 in wire mesh cages. Food and water were given ad libitum.

All diets contained a level of fat supplying 35.4% of total fatty acids. The ratio of unsaturated to saturated fatty acid in the diets was varied from zero (diet containing only saturated fatty acid supplied as acetylated monoglycerides) to 3.0 (all fatty acids supplied as a one to one mixture of corn oil and lard). Intermediate ratios were obtained by various combinations of these fat sources (table 1). In addition to the fat moiety, the diets contained the following in per cent composition: vitamin-test casein,[4] 20; B-vitaminized casein, 4 (Herting et al., '55); U.S.P. XIV salts, 5; and 40,000, 4,000 and 510 I.U. of vitamins A, D and E, respectively, per kilogram of diet. Glucose[5] made up the difference between the total of the above ingredients and 100%.

The number of rats killed at each 8-week interval varied according to the number left in each group, but rats were chosen whenever possible to leave essentially unchanged the average body weight of those remaining. Pieces of fat attached to adrenal, kidney, stomach, testis or ovary and abdominal wall were fixed with Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Two sections from 10 to 30 μ apart were taken from each tissue.

Sections were identified by code number and not correlated with dietary groups until after completion of microscopic examination. After examining with a scanning microscope, the amount of fat attached to tissue or organ was classified as "slight," "moderate," or "abundant," according to whether it filled one or less, one to two, or two to three fields, respectively, using a 3 × objective lens with a 10 × eyepiece. A high-powered objective lens was then used to examine foreign-body reaction and to count foci of reaction. Involvement ranging from a single cell to a group of adjoining cells was considered a focus of reaction. An arbitrary rating of severity was made by dividing the number of foci of reaction by the number of fields of fat.

RESULTS

Examination of fat adhering to various tissues showed that incidence of lipogranuloma was greater in fat associated with testis or ovary than with any of the other tissues (table 2). Severity, however, showed no correlation with location of fat in the body. Incidence of lipogranuloma in males and females was 39% and 36%, respectively; consequently these observations are combined.

[4] General Biochemicals, Inc.

[5] Cerelose Dextrose 2001, obtained from Corn Products Refining Company.

TABLE 2

Incidence of lipogranuloma in fat associated with various tissues from rats on various diets[1]

Group	UFA/SFA[2]	Incidence in fat			
		Perirenal-periadrenal[3]	Perigonadal	Perigastric	Subcutaneous
1	0.0	19	43	26	35
2	0.17	35	87	57	39
3	0.33	24	74	39	27
4	0.60	11	22	9	6
5	1.0	0	5	0	3
6	1.5	3	3	0	0
7	3.0	0	0	2	0
Total rats examined		252	250	234	246
Incidence (number of rats)		30	76	40	34
Incidence (%)		11.9	30.4	17.1	13.8

[1] 28 to 42 rats per group.

[2] Ratio of unsaturated fatty acid to saturated fatty acid. Constant level of 35.4% of fatty acid in diet.

[3] Combined because of proximity.

TABLE 3

Relationship between various proportions of unsaturated to saturated fatty acids in the diet and the incidence and severity of lipogranuloma

Group	UFA/SFA[1]	Time	Rats	Reaction[2] in perigonadal fat			
				None	Slight	Moderate	Severe
		weeks					
1	—	2	7[3]	5	0	0	0
		4	6[3]	4[4]	0	0	0
		6	4[5]	1[6]	0	2	0
		8	5	1[7]	0	2	2
		16	6	2	0	4	0
2	0.17	8	6	3[4]	2	1	0
		24	6	0	1	5	0
		49	2	0	0	1	1
3	0.33	8	8	4[8]	2	2	0
		24	8	3[9,10,11]	2	3	0
		49	4	0	3	1	0
4	0.60	8	8	8	0	0	0
		24	8	6	1	0	1
		49	4	2	2	0	0
5	1.0	8	8	8	0	0	0
		24	8	8	0	0	0
		49	6	4	2	0	0
6	1.5	8	8	8	0	0	0
		24	8	8	0	0	0
		49	5	5	0	0	0
7	3.0	8	8	8	0	0	0
		24	8	8	0	0	0
		49	5	5[9]	0	0	0

[1] Ratio of unsaturated fatty acid to saturated fatty acid. Constant level of 35.4% of fatty acid in diet.

[2] "Slight" means one or less focus of lipogranuloma per field of fat. "Moderate" means one to 5 foci of lipogranuloma per field of fat. "Severe" means more than 5 foci of lipogranuloma per field of fat.

[3] Gonadal fat not available for two rats, one of which shows no reaction in other sections and one of which shows "slight" in subcutaneous fat.

[4] One rat shows "slight" in subcutaneous fat.

[5] Testicular fat not available for one rat which shows "slight" in perirenal fat.

[6] Shows "slight" in perirenal and in subcutaneous fat.

[7] Shows "moderate" in perigastric fat and "slight" in subcutaneous fat.

[8] One rat shows "slight" in perirenal fat.

[9] One rat shows "slight" in perigastric fat.

[10] One rat shows "moderate" in perigastric fat.

[11] One rat shows "severe" in perirenal, perigastric, and subcutaneous fat.

Detailed results showing the incidence and severity of lipogranuloma in peri gonadal fat are summarized in table 3. Rats were killed at two-week intervals in group 1 because of their high early mortality, to determine how early the lesion develops. The reaction appeared within two weeks in one of 7 rats. Cox and DeEds ('58) have reported that the reaction appears as early as 30 days.

Under the conditions of this experiment, lipogranuloma decreased in incidence and severity as the ratio of unsaturated fatty acid to saturated fatty acid increased. At a ratio of 1.0 or greater, lipogranuloma was negligible. The few cases observed at levels of 50% of unsaturated fatty acid or greater, were those with single isolated cells, and may represent normal biological variation due to local conditions at the cellular level. A single involved cell was also seen in another experiment in a rat which was fed lard for 24 weeks, followed by corn oil for 16 weeks (Herting et al., '59).

DISCUSSION

The evidence thus far accumulated indicates that the foreign-body type of reaction is the result of a relative excess, rather than an absolute quantity, of saturated fatty acid in the diet. The reaction has not been observed in rats fed diets containing 65% of lard or 50% of distilled acetylated monoglycerides made from lard, supplying 24% and 12%, respectively, of the diet as long-chain saturated fatty acids;[6] nor was it seen to any extent in groups 5, 6, and 7 in which up to 17.6% of the diet was saturated fatty acid. In each of these diets, unsaturated fatty acids equalled or exceeded 50% of total fatty acids.

In contrast, Cox and DeEds ('58) found occasional lipogranuloma with a diet containing as little as 3.5 to 4.0% of saturated fatty acids, but also containing only 4.5 to 6.0% of *fat* other than saturated acetylated monoglycerides.[7] The unsaturated to saturated fatty acid proportion was approximately one to one under these conditions, and an occasional lipogranuloma was observed after long periods of time. Thus, an excess of saturated fatty acids, relative to the amount of unsaturated fatty acids (namely, nutritional deficiency of unsaturated fatty acid), is probably the determining factor. These data also suggest that the effect of a given proportion of unsaturated to saturated fatty acids is approximately the same, whatever the total fat level in the diet.

Some natural fats contain more than 50% of saturated fatty acids and might be expected to produce lipogranuloma if fed as the sole source of dietary fat. Cox and DeEds ('58) have reported that neither butter nor tallow, at levels up to 50% of the diet, caused lipogranuloma. These fats, with unsaturated to saturated fatty acid ratios, ranging from 0.65 to 0.90, were fed for 30 days.[7] On the basis of our results (table 3), this period of time would be insufficient for the reaction to develop with this ratio of unsaturated to saturated fatty acid. Another factor, arguing against butter as a causative agent within this period of time, is that short-chain, saturated fatty acids probably do not induce lipogranuloma (Herting et al., '59).

How serious are these foreign-body reactions in adipose tissue? All evidence so far indicates that lipogranuloma is a morphological abnormality, but that the affected tissue does not impair overall biologic performance of the rat. For example, easy and complete reversibility of the cellular reaction (Herting et al., '59) has indicated that no permanent change occurred. In addition, chronic toxicity studies with rats fed high levels of saturated fat for long periods (Ambrose et al., '58a, '58b), showed that reproduction and lactation were normal as long as adequate amounts of α-tocopherol and essential fatty acids were supplied. Weight gain was slightly slower than in groups fed comparable levels of fat of higher absorbability, probably because of the difference in available calories and not because of the presence of lipogranuloma. Whether the foreign-body reaction within the fat cell has deleterious effects on metabolism of the cell itself deserves attention.

What is the significance in human nutrition of these findings on production and prevention of lipogranuloma in the rat? Although fat lesions described in some

[6] D. C. Herting and R. C. Crain, unpublished data, 1956.

[7] F. DeEds, personal communication.

pathological conditions (sclerema neonatorium, traumatic fat necrosis, and others) resemble lipogranuloma in the rat, this foreign-body type reaction has not been reported to occur in "normal" human adipose tissue. However, more comprehensive histological surveys may show that it occurs.

Average fat consumption figures for the United States (USDA, '57) indicate that 60 per cent of the daily intake of long-chain fatty acids is unsaturated. Consumption of diets with this dietary ratio of 1.5 (unsaturated to saturated fatty acids) should theoretically not induce lipogranuloma formation, assuming, of course, that the composition of human depot fat responds to dietary fat in the same manner and degree as that of the rat. Certain persons may, at least temporarily, ingest fat with lower ratios and develop lipogranuloma, but the cell reaction should disappear when dietary intake of unsaturated to saturated fatty acids returns to a ratio greater than 1.0 (Herting et al., '59).

SUMMARY

Occurrence of lipogranuloma, a foreign-body type reaction in adipose tissue, has been studied in rats fed diets containing ratios of unsaturated to saturated fatty acid ranging from zero (diet containing only saturated fat as acetylated monoglycerides made from hydrogenated lard) to 3.0 (diet containing a mixture of equal parts of corn oil and lard).

The earliest observed occurrence of lipogranuloma was at two weeks, on the diet containing completely saturated fat. Fat-cell reaction did not appear to a significant degree when the ratio of unsaturated to saturated fatty acid was 1.0 or greater, but it increased in incidence and severity as the ratio decreased below 1.0.

ACKNOWLEDGMENT

The authors express their appreciation to Marion Ludwig and William Swanson for assistance in the care of the animals, to Dr. Robert Roudabush and William Uskavitch, Eastman Kodak Medical Laboratory, for advice and preparation of the slides, and to Emma Drury and Sharon Kinney for technical assistance with this work.

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The Effect of Diet on Growth Rate and Feed Efficiency in the Normal Rat[1,2]

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This study was undertaken to evaluate the effect of the level of fat, the caloric density, and the amount of protein in the diet, on the rate of body weight gain and caloric efficiency of feed utilization in the male weanling rat. Yoshida et al. ('57, '58) reported that the dietary fat level did not affect the caloric efficiency of feed utilization in short term feeding experiments, even though caloric intakes and rates of body weight gain increased with increasing dietary fat levels, when rations with constant protein:calorie ratios were fed.

In the longer-term studies reported here, the level of fat and caloric density of the ration were correlated positively with the caloric efficiency of feed utilization and with the rates of body weight gain observed. These results appear to confirm earlier reports of studies using the chick (Siedler et al., '55; Rand et al., '58) and the dog (Siedler and Schweigert, '52). The experimental rations used in the present studies were based on casein, sucrose, and either lard or corn oil, and were designed to cover a broad range of protein:calorie ratios.

EXPERIMENTAL METHODS

Male weanling rats were fed a stock diet[4] for three days. Each experimental diet was fed to a group of 14 animals. Groups were equalized by weight; animals were placed in individual cages having raised, screen floors and placed on experiment when 25 days old. Food and water were provided ad libitum during the 8-week experimental period.

Animals were weighed bi-weekly and gross food intake measured. With the diets and methods used, there was practically no food spillage and calculations of average grams of body weight gain observed per 100 Cal. were therefore based upon gross food intake.

The composition of the experimental rations is shown as ingredient percentages by weight and by calculated calories in tables 1 and 2. Vitamin and mineral supplements were provided in a constant ratio to calculated calories in all rations, with ration no. 1 supplemented according to Lushbough et al. ('57), and all other rations supplemented appropriately according to calculated caloric content.

Rations 1 through 4 provided increasing levels of casein at a low fat level. Rations 5 through 8 provided the same increasing levels of casein on a calculated calorie basis, in combination with 4 times as many calories from fat, as did rations 1 to 4, respectively. Rations 9 and 10 represented dilution diets, in which 15 parts of fat were added per 100 parts of rations no. 1 and 3, respectively, with appropriate adjustments to maintain constant ratios of vitamins and minerals to calories. Similarly, rations no. 11 and 12 were dilution diets in which 33.75 parts of sucrose, calorically equivalent to the 15 parts of added fat in rations 9 and 10, were added per 100 parts of rations no. 1 and 3.

For comparative purposes, lard and corn oil were included in the simultaneous, par-

Received for publication August 31, 1959.

[1] A preliminary report based on the results given was presented at the annual meeting of the American Institute of Nutrition, 1958 Federation Proc., *17:* 482 (abstract).

[2] Journal paper no. 188, American Meat Institute Foundation.

[3] Present address: Research Division, Mead Johnson & Co., Evansville, Indiana.

[4] Ralston-Purina Laboratory Chow.

allel experiment series, and each fat was evaluated in each ration, except for the omission of corn oil from rations no. 2, 4, 6 and 8.

RESULTS

Experimental results observed in these studies are given in table 3. When low levels of fat were fed, average body weight gains, and average body weight gains per 100 Cal. of gross feed intake increased progressively with increasing levels of casein in the rations (table 3, rations no. 1, 2, 3 and 4).

In rations no. 5, 6, 7 and 8, lard or corn oil provided 4 times as many calories as in rations no. 1, 2, 3 and 4, while the protein: calorie ratios were identical in the respective diets of the two series of rations (table 2). Feeding the higher fat levels resulted in further small increases in the caloric efficiency of feed utilization for each of the levels of casein tested, with the single exception of group 5, fed corn oil.

Similarly, rates of body weight gain were higher when the higher levels of fat were fed, for all groups except group 8 fed lard, when compared with the respective low fat diets in groups 1 to 4. Figure 1 shows body weight gains achieved at 2, 4, 6 and 8 weeks, by animals fed the lard-containing rations no. 1, 3, 5 and 7. The results given in this figure for each of the time intervals indicates the smooth response and the consistent differences observed for the rations indicated.

The results observed with the animals fed rations containing corn oil were closely similar, as may be inferred from the data in table 3. No figure is therefore shown for these animals.

From these results, it may be concluded that when low levels of fat were fed, as in rations no. 1, 2, 3 and 4, progressive increases in protein: calorie ratios produced progressive improvements in rates of body weight gain and caloric efficiency of feed utilization. The greatest effects were observed when casein was increased from 11.8 to 23.6% of the calculated calories (ration no. 1 vs. no. 2), with smaller increments noted for further similar increases in protein: calorie ratios. When lard or corn oil provided 44.2% of the calculated calories (rations no. 5, 6, 7 and 8), further small increases in average body weight gain and weight gain/100 Cal. of gross food intake were observed, when compared with the results for rations no. 1, 2, 3 and 4, in which fat provided 11.1% of the calories.

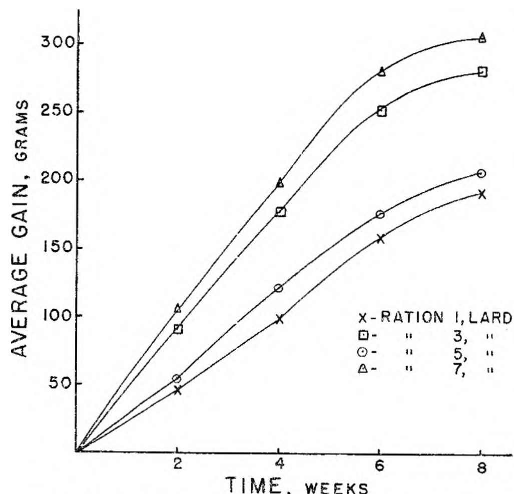

Fig. 1 Effect of increasing percentages of calories from casein and lard upon body weight gains.

In the dilution diets, rations no. 1 and 3 were diluted by the addition, per 100 parts, of 15 parts of fat (rations no. 9 and 10), or 33.75 parts of sucrose (rations no. 11 and 12), respectively, while vitamin: mineral: calorie ratios were held constant. The feeding of ration no. 9 resulted in significant decreases in average body weight gain and caloric efficiency of feed utilization at the low casein level, when compared with the results observed for ration no. 1. When ration no. 10 was fed, however, no significant differences were observed, compared with ration no. 3. Similarly, weight gains and caloric efficiency were depressed still further when the rations were diluted with sucrose at the low casein levels in group 11, as compared with groups 1 and 9, while the differences among groups 3, 10 and 12 with the higher casein levels were all relatively small.

The differences in body weight gain achieved when these rations were fed are shown graphically in figure 2. From these results, the major effects due to ration dilution with lard or corn oil, or sucrose, may be ascribed apparently to reductions

TABLE 1

Percentage composition of experimental rations by weight[1]

Ration no.	1	2	3	4	5	6	7	8	9	10	11	12
Casein	12	24	36	48	14.8	29.5	44.3	59.1	10.3	30.9	8.9	26.6
Lard or corn oil	5	5	5	5	24.6	24.6	24.6	24.6	17.2	17.2	3.7	3.7
Sucrose	78.5	66.5	54.5	42.5	55.0	40.2	25.5	10.7	67.3	46.7	83.0	65.2

[1] Vitamins, minerals and fish liver oil were provided in a constant ratio to calculated calories, to make 100% (Lushbough et al., '57).

TABLE 2

Calculated percentage composition of experimental rations by calories[1]

Ration no.	1	2	3	4	5	6	7	8	9	10	11	12
Casein	11.8	23.6	35.4	47.2	11.8	23.6	35.4	47.2	8.9	26.6	8.9	26.6
Lard or corn oil	11.1	11.1	11.1	11.1	44.2	44.2	44.2	44.2	33.2	33.2	8.3	8.3
Sucrose	77.1	65.3	53.5	41.7	44.0	32.2	20.4	8.6	57.9	40.2	82.8	65.1
Calories/100 gm	407	407	407	407	501	501	501	501	465	465	401	401

[1] Calculated as 4 Cal./gm for casein and sucrose, 9 Cal./gm for lard or corn oil.

TABLE 3

Body weight gain and caloric efficiency of feed utilization in 8-week test period

Ration no.	1	2	3	4	5	6	7	8	9	10	11	12
Lard												
Average gain, *gm*	192	264	281	295	209	292	306	276	150	276	104	276
Average gain, gm/100 Cal. gross food intake	5.5	7.0	7.2	7.5	5.7	7.3	7.7	7.7	4.7	7.3	3.8	7.0
Corn oil												
Average gain, *gm*	188	—	260	—	207	—	297	—	123	292	97	265
Average gain, gm/100 Cal. gross food intake	5.7	—	7.2	—	5.1	—	7.5	—	3.4	7.2	3.6	7.3

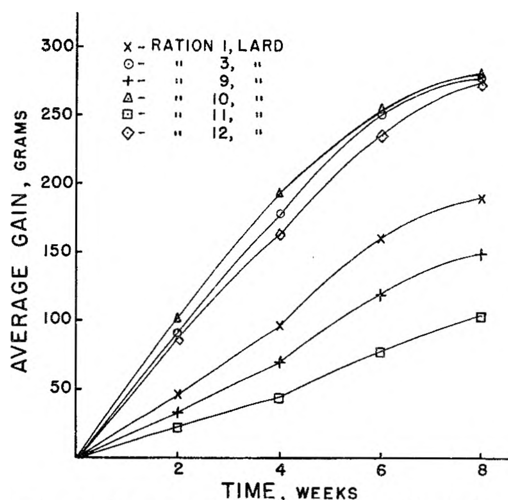

Fig. 2 Effects of ration dilution with lard or sucrose upon body weight gain.

in the protein:calorie ratios for the rations studied, since such reductions assumed much greater nutritional significance at the low, growth-limiting levels of casein used in rations no. 9 and 11, than in rations no. 10 and 12.

Comparing the results for the feeding of each of the fats tested (table 3), average body weight gains were slightly higher for the animals fed lard than for those fed corn oil, in all groups except those fed ration no. 10. Although several small differences were observed for caloric efficiency of feed utilization, these were not consistent and not statistically significant.

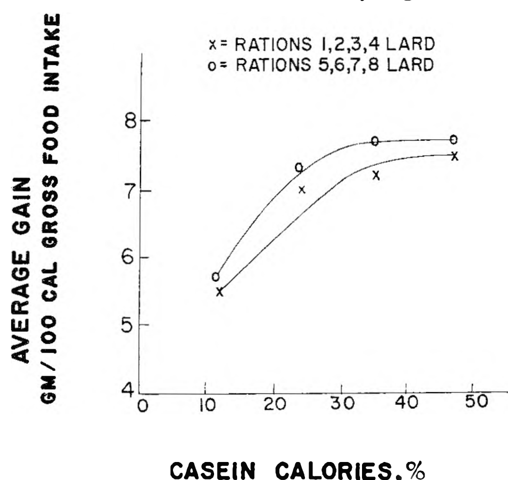

Fig. 3 Effect of increasing casein calories upon body weight gain.

Thus, no overall effect of the specific fat fed can be attributed to either the lard or the corn oil.

When casein provided increasing percentages of the calories in the experimental rations, the average body weight gains/100 Cal. increased rapidly between the two lower casein levels fed, with evidence of a plateau between the two higher casein levels (fig. 3). Thus, although increasing levels of casein in the diet stimulated increased rates of body weight gain at each of the levels fed in this experiment, the efficiency of protein utilization to support body weight gain decreased rapidly at the higher casein levels. This effect is to be expected at some high protein intake level, in view of the known deleterious effects of feeding extremely high levels of protein (90% of calories) to young animals. The curves shown in figure 3 emphasize further the effects of providing increased percentages of the calories from fat, upon the rates of body weight gain observed.

DISCUSSION

Rand et al. ('58) reported results similar to those given here, in studies of the rapidly growing chick, when feed was given ad libitum or equalized among the several experimental groups. Although Yoshida et al. ('57) reported that with increases in dietary fat from approximately 2 to 50% of calories, results similar to those reported here were obtained when sucrose was used as the dietary carbohydrate, these workers subsequently reported ('58) that with rations providing 10 to 14% of the calories from protein, the types of dietary carbohydrate fed affected significantly the results observed at the low fat levels tested (2 and 21% of calories), but not at the high fat level (50% of calories). Thus, when dextrin was fed, values for body weight gain and caloric efficiency of feed utilization were similar, regardless of the dietary fat level; while the feeding of sucrose or dextrose resulted in increasing rates of body weight gain but in no change in caloric efficiency of feed utilization, with increasing amounts of fat in the diet, during the two-week test period.

In the present long-term growth studies, progressive increases in protein:calorie ra-

tios over a broad range (11.8 to 47.2% of calories from casein) produced consistent increases in average body weight gain and in the caloric efficiency of feed utilization, when either lard or corn oil provided 11.1 or 44.2% of the calories in the experimental rations, and sucrose was used as the dietary carbohydrate. No significant differences in response to the two fats tested were observed, and this result further supports the observations of Thomasson ('55) and Deuel et al. ('47).

When these experimental rations were diluted with either of the fats studied or with sucrose, the biological performance of the experimental animals was consistently reduced, with casein fed at growth-limiting levels. Such effects should be carefully considered in the design of nutritional experiments in order to assure that the results observed may be ascribed to the specific nutrients being fed.

SUMMARY AND CONCLUSIONS

Long-term studies of the effect of the level of dietary protein and fat on the rate of body weight gain and on the caloric efficiency of feed utilization in male weanling rats have been described. When rations based on casein, sucrose and lard or corn oil were fed ad libitum, the rate of body weight gain and the caloric efficiency of feed utilization increased with progressive increases in protein:calorie ratios, and further increases were observed for each protein:calorie ratio studied, when the fat:calorie ratio was increased by a factor of 4.

Dilution of the experimental rations by the addition of either fat or sucrose resulted in decreased response and efficiency when protein was limiting for growth, but such dilution was without effect at higher protein levels.

No significant differences in response to the feeding of lard or corn oil were observed.

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Diet-Type (Fats Constant) and Blood Lipids in Man[1]

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It is now well established that the concentration of lipids, especially cholesterol and beta lipoproteins, in the blood of man is markedly influenced by the fats in the diet. Other nutrients in the diet of some experimental animals have been reported to affect the blood lipid concentration, especially in young, growing animals maintained on extremes of deficiency or excess of one or another nutrient. In adult man, there is no acceptable evidence that, in calorie equilibrium and with proper control of other factors, variations within the limits of natural diets in nutrients other than the fats are influential.

Nevertheless, there have been speculations (Portman and Stare, '59) that dietary characteristics other than fat content may contribute to the striking differences (Keys, '52, '57; Keys, et al., '58) in serum cholesterol levels observed in comparisons between populations. Walker and Arvidsson ('54; also Bersohn, et al., '56) have suggested that the high fiber content or "bulk" of the diet of some Bantu natives may be partly responsible for the characteristically low serum-cholesterol levels in those people in South Africa. In our own studies, as will be seen below, we have seen serum cholesterol levels somewhat lower than might have been predicted from the relationships between dietary fats and serum cholesterol observed in controlled experiments on man (Keys, Anderson and Grande, '59). The diets of the populations involved in these cases are characterized by large quantities of fruits and vegetables. Such diets tend to be high in complex carbohydrates, such as pectins, hemicelluloses and fiber, and in sugars other than sucrose. Accordingly, we have performed controlled dietary experiments in man to see whether these factors are influential. The present paper reports the results of comparisons between two general kinds of diets differing in type of carbohydrate. One, which we have termed the "American type" is relatively low in fruits and fresh vegetables and high in sucrose as compared with the other, the "Italian type." Both low and moderate fat versions of each type were used. Calorie balance was maintained on all diets and the attempt was made to assure comparability of protein and vitamin as well as fat content of the diet compared.

METHODS

Subjects

Twenty-eight male schizophrenic patients of the Hastings State Hospital from 41 to 63 years old and between 66 and 115% of average body weight for height and age were the subjects. They were clinically normal in physical health and none had erratic eating habits. Subjects were divided into 4 sub-groups of 7 men each, matched as to age, relative body weight and degree of habitual physical activity. Body weights in night clothing were measured once a week before breakfast and calorie intakes adjusted by changing the allowance of bread and jelly in individual rations to keep weights constant.

Total cholesterol in the fasting serum of each man was determined on 4 occasions during each of the two 6-week dietary periods, twice near the middle of the period and twice near the end, using the method of Abell et al. ('52) as modified

Received for publication August 31, 1959.
[1] The work reported here was aided by research grants from the United Fresh Fruit and Vegetable Association, Washington, D. C., the National Dairy Council, Chicago, the American Heart Association, New York, the Minnesota Heart Association, the Winton Companies Fund, Minneapolis and the Schweppe Foundation, Chicago.

in this laboratory (Anderson and Keys, '56). Phospholipids and the combined weight of the total fatty acids and cholesterol were determined in pooled samples made by combining equal volumes of serum from three men from the blood drawn on two days at the middle of each diet period. Each group of 7 men provided two pools in each period. One pool included the three men highest in serum cholesterol (at the first test), the other pool included the three men lowest in the cholesterol and the middle man was omitted.

The phospholipid determination was done on 0.100 ml of serum by shaking with 4 ml of chloroform-methanol mixture (2 to 1), filtering, washing and evaporating to dryness in a hard glass test tube of about 50 ml capacity. A 0.50 ml portion of 60% of perchloric acid was added and the lower end of the tube was inserted in a metal socket heated so as to bring the contents to 220°C. (Perchloric acid fumes were drawn away through an all-glass system to a filter pump in order to avoid danger of explosion.) After 5 minutes of digestion the solution was colorless and after 10 minutes it was cooled. Using a syringe pipet, 3.3 ml of amidol solution (1.5 gm/l) containing also $NaHSO_3$ (30 gm/l) was added. After mixing 3.3 ml of ammonium molybdate solution (6 gm/l) were added in the same way and the contents again mixed. Absorbance readings were made with 660 millimicron light. This is the extraction method used by Folch et al. ('57) and by Sperry ('55) and reported by the latter author to remove all the lipid phosphorus from serum and no other compounds containing phosphorus. The color development method is that of Allen ('40).

For the determination of total fatty acids and cholesterol, 1 ml of serum, 1 ml of KOH (330 gm/l) and 8 ml of absolute ethanol were mixed in a 40 ml glass-stoppered tube and incubated at 37 to 46°C for 90 minutes. After this saponification, 5 ml of water, 1 ml of concentrated HCl and 9 ml of petroleum ether (60 to 80°C b.p.) were added, the tube was shaken briefly and after the layers had separated, most of the petroleum ether was transferred by a capillary pipet to a 50-ml Erlenmeyer flask. The extraction was repeated with 6 ml of petroleum ether. Care was exercised to avoid transferring any of the water layer. The flask was heated to 60°C and nitrogen passed into it until it appeared dry. It was then heated in a vacuum oven at 60 to 65°C while the pressure was reduced to 100 mm of mercury in 5 to 10 minutes. Dry air was admitted to the oven to bring the pressure to atmospheric in about 2 minutes, the flask was polished with a towel, let stand near the balance for 30 minutes and weighed to the nearest 0.01 mg. An 8-ml portion of petroleum ether was swirled around in the flask and poured out taking care to leave any visible particles in the flask. This was repeated twice with 4-ml portions. The flask was drained upside down until it appeared dry and then dried in the vacuum oven and weighed as before. With each set of flasks two empty flasks were included which were rinsed, heated, wiped and weighed like the others. Assuming that the weight changes in these flasks were due to moisture on the glass surface, corrections were computed and applied to the weights of the flasks containing samples.

Phospholipids were computed as 25 times lipid phosphorus. Triglycerides were computed by the formula $G = 1.046\,(TFAC) - 1.586\,(SC) - 0.738\,(PL)$ in which the units are mg per 100 ml, TFAC is the sum of total fatty acids and cholesterol, SC is total serum cholesterol and PL is phospholipids. This formula depends on the assumptions that 73% of cholesterol is esterified, that each molecule of phospholipid contains one P atom and two fatty acid residues, and that the average molecular weight of the fatty acids in all fractions is 273.

The TFAC lipid regularly contains phosphorus equivalent to about 5 mg of phospholipid per 100 ml of serum. This probably is unhydrolyzed phospholipids such as sphingomyelin. The method of TFAC was applied to serum omitting the HCl and the unsaponifiable and unsaponified lipids were weighed and analyzed for phosphorus and for cholesterol. The weight was about 15 mg per 100 ml greater than the cholesterol of which about one-third was apparently phospholipid, judging by the phosphorus content. The indication

is that the TFAC determinations are about 13 mg per 100 ml too high due to the presence of unexplained unsaponifiable lipid and the non-faty acid portions of unsaponified phospholopids. For this reason the computed triglyceride (G) values are probably high by about 14 mg per 100 ml.

Diets

The diets were prepared and served by quantitative methods. Food rejected and adjustments of bread and jelly were recorded for each man who otherwise ate the standard serving. Precautions were taken to keep unauthorized food from the subjects.

Diet IL (Italian low fat) was characterized by large amount of bread, macaroni, beans and other leguminous seeds, fresh fruits and leafy vegetables and by small portions of meat, cheese and eggs. Diet AL (American low fat) was planned to be the same in total protein, fat and digestible carbohydrate and nearly the same in the proportion of fat derived from plants, from milk and from other animal sources but contained more sucrose, skim milk and meat.

TABLE 1A

The raw foods of one serving of diet IL for a typical day

	Raw food	Calories	Protein	Total fat	Carbohydrate	
					Fiber	Non-fiber
	gm		*gm*	*gm*	*gm*	*gm*
Meats						
Beef, ground	15	27	2.9	1.6	—	—
Ham, smoked, cured	25	97	4.2	8.8	—	0.1
Lard	3.1	28	—	3.1	—	—
Eggs	48	77	6.1	5.5	—	0.3
Milk products						
Butter	1.2	9	—	1.0	—	—
Cheese, parmesan	10	39	3.6	2.6	—	0.3
Dry skim milk	2.2	8	0.8	—	—	1.5
Whole milk, fluid	180	122	6.3	7.0	—	8.8
Sugar, white or brown	45.9	184	—	—	—	45.9
Cereals						
Cornmeal	30	109	2.4	0.4	0.2	23.3
Wheat flour, white	295.5	1076	31.0	3.0	0.9	224.0
Legumes						
Lima beans	40	133	8.3	0.5	1.7	22.9
Split peas	40	138	9.8	0.4	0.5	24.2
Yeast (in bread)	4.2	4	0.4	—	—	0.5
Potatoes and roots						
Onions	120	54	1.6	0.2	1.0	11.4
Potatoes	200	166	4.0	0.2	0.8	37.4
Radishes	20	4	0.4	—	—	0.5
Green and leafy vegetables						
Lettuce	100	15	1.2	0.2	0.6	2.3
Romaine	100	15	1.2	0.2	1.0	1.9
Spinach, fresh	200	40	4.6	0.6	1.2	5.2
Fruits and other vegetables						
Apples, fresh	300	174	0.8	1.2	3.0	41.6
Apples, vacu-dry	50	185	0.7	1.2	2.4	40.4
Oranges, fresh	300	136	2.8	0.6	1.8	31.8
Cucumbers	25	3	0.2	—	0.1	0.6
Tomatoes, fresh	50	10	0.5	0.2	0.3	1.7
Tomato paste	60	57	2.9	0.8	0.5	10.8
Squash, winter	200	76	3.0	0.6	2.8	14.8
Olive oil	17	153	—	17.0	—	—
Total		3139	99.5	56.9	19.0	572.4

In regard to protein, diet IL provided more from legumes and to a small degree from fruits and green leafy vegetables and diet AL provided more from meat and milk products. Carbohydrates in diet IL were provided more from fruits, legumes, and green leafy vegetables than in diet AL which emphasized more sugar and milk products. A complete list of the raw foods used in each diet in a typical day is given in table 1. Seven different menus of similar pattern were used in rotation.

Diets IM (Italian moderate fat) and AM (American moderate fat) were prepared from the corresponding low fats diets by omitting 225 gm of the low-fat bread and adding 54 gm of olive oil and 20 gm of soy protein flour.[2] This substitution increased the percentage of calories supplied by fat from 16 to 31% with an equal decrease in carbohydrate and only a small exchange (16.4 gm or 2.1% of calories) of wheat protein for soy protein. The 54 gm of olive oil used comprised (as glycerides) 7 gm of saturated, 40 gm of monoene, and 7 gm of polyene fatty acids.

[2] C-1 Assay Protein, Archer Daniels Midland Co., Cincinnati.

TABLE 1B

The raw foods of one serving of diet AL for a typical day

	Raw food	Calories	Protein	Total fat	Carbohydrate	
					Fiber	Non-fiber
	gm		*gm*	*gm*	*gm*	*gm*
Meats						
Beef, round	75	136	14.6	8.2	—	—
Beef, ground	50	91	9.8	5.5	—	—
Eggs	57	91	7.2	6.5	—	0.4
Milk products						
Butter	9.4	68	—	7.6	—	—
Dry skim milk	66.5	241	23.6	0.8	—	34.3
Sugar, white or brown	75	285	—	—	—	71.3
Jelly	60	152	0.2	—	—	39.0
Corn syrup	56	161	—	—	—	41.6
Cereals						
Farina	37	136	4.0	0.3	0.1	28.4
Wheat flour, white	214.8	783	22.5	2.2	0.6	163.0
Macaroni	24	90	3.1	0.3	0.1	18.2
Rice	37	134	2.8	0.1	0.1	29.3
Legumes						
Green peas, canned	100	91	4.5	0.6	2.3	14.9
Yeast (in bread)	2.9	2	0.3	—	—	0.4
Potatoes and roots						
Carrots	121	51	1.5	0.4	1.4	9.9
Onions	53	24	0.7	0.1	0.4	5.0
Potatoes	200	166	4.0	0.2	0.8	37.4
Green and leafy vegetables						
Celery	52	9	0.1	—	0.3	1.6
Lettuce	60	10	0.8	0.1	0.4	1.6
Fruits and other vegetables						
Apples, fresh	65	38	0.2	0.3	0.6	9.0
Apples, vacu-dry	24	87	0.4	0.6	1.2	18.9
Oranges, juice	12	6	0.1	—	—	13.0
Orange-grapefruit sections	100	40	0.6	0.2	0.2	9.9
Raisins	32	87	0.7	0.2	—	23.0
Tomato juice	100	21	1.0	0.2	0.2	4.1
Olive oil	20.8	187	—	20.8	—	—
Totals		3187	102.7	55.2	8.7	562.5

TABLE 2

Average quantities of nutrients eaten per man per day

	Diet			
	IL	AL	IM	AM
Calories	3040	3030	3100	3090
Proteins, *gm*	92	97	93	103
Total fat, *gm*	55	55	104	105
Saturated fat, *gm*	23	19	30	27
Monoene fat, *gm*	26	30	65	67
Polyene fat, *gm*	6	6	9	11
Total carbohydrate, *gm*	547	551	465	450
Fractions of carbohydrate:				
Fiber, *gm* CHO	19	6	18	6
Sucrose, *gm* CHO	78	181	82	157
Milk, *gm* CHO	12	36	12	38
Potatoes and root vegetables, *gm* CHO	51	58	47	55
Cereals, *gm* CHO	198	193	121	121
Legumes, *gm* CHO	44	9	43	9
Leafy vegetables, *gm* CHO	17	4	16	3
Fruits and other vegetables, *gm* CHO	128	64	126	60
Cholesterol, *mg*	330	460	320	450
Groups of men	WX	WX	YZ	YZ

The composition of the diet eaten (table 2) was computed from the raw ingredients using food tables except for the saturated, monoene and polyene fractions of the fat. These latter fractions were computed by applying to the values for total fat derived from the food tables the values for percentage composition of the methyl esters of the fatty acids of the diet as determined by gas chromatography. The samples for gas chromatographic analysis were obtained by collecting one standard serving of each food each day for 7 days, grinding the mass without loss, mixing well, homogenizing a portion, extracting lipids by the method of Soderhjelm and Soderhjelm ('49), saponifying, extracting fatty acids from the acidified 50% alcohol into petroleum ether and converting the fatty acids to methyl esters by treating with diazomethane in ether.

RESULTS

Serum cholesterol

The serum cholesterol values are presented in table 3. Each cholesterol concentration value is the mean for 4 serum samples, each analyzed in duplicate. Two of these serum samples were taken three weeks after the diet started and the others three weeks later. There was no tendency toward a change in the cholesterol values from the third to the 6th week on the same diet. The data for groups W and X indicate clearly that when the men were subsisting on diet IL they had lower serum cholesterol values than when on diet AL. Similarly the data for groups Y and Z show that diet IM resulted in lower serum cholesterol values than diet AM. Each of the 4 groups and all but one of the 28 men showed cholesterol changes in the same direction, the average difference being 17 mg per 100 ml.

Although the attempt had been made to equalize the amount and nature of fat in diets IL and AL and in diets IM and AM, there were small differences in the fat in the diets as actually consumed as shown in table 2. The expected differences in serum cholesterol due to dietary fat differences have been well investigated in this laboratory and are best predicted by equation 2c of Keys, Anderson and Grande ('59). The results of the calculation with this equation are shown in table 4. The slightly larger amount of saturated fat in diet IL than in AL and in IM than in AM, would make an average predicted difference in serum cholesterol of about 3 mg per 100 ml in the direction *opposite* to that observed. The serum cholesterol dif-

TABLE 3

Average serum cholesterol concentration for each subject in each period and the differences (Δ cholesterol) caused by changing diet, mg per 100 ml

The diet designations are above the data for each group. Diets IL and IM were high in fruits and vegetables, diets AL and AM were high in sucrose and skim milk.

Subject		Periods		Δ cholesterol
		1	2	
	Diet	IL	AL	AL − IL
W1		167	172	+ 5
W2		171	181	+ 10
W3		144	150	+ 6
W4		200	229	+ 29
W5		166	185	+ 19
W6		139	160	+ 21
W7		177	189	+ 12
Mean for group W		166	181	+ 15
	Diet	AL	IL	AL − IL
X1		131	120	+ 11
X2		176	148	+ 28
X3		184	154	+ 30
X4		138	118	+ 20
X5		142	126	+ 16
X6		236	240	− 4
X7		156	137	+ 19
Mean for group X		166	149	+ 17
	Diet	IM	AM	AM − IM
Y1		185	193	+ 8
Y2		146	159	+ 13
Y3		206	222	+ 16
Y4		149	166	+ 17
Y5		183	200	+ 17
Y6		190	234	+ 44
Y7		198	209	+ 11
Mean for group Y		180	198	+ 18
	Diet	AM	IM	AM − IM
Z1		209	183	+ 26
Z2		148	139	+ 9
Z3		204	176	+ 28
Z4		188	174	+ 14
Z5		158	120	+ 38
Z6		202	194	+ 8
Z7		190	176	+ 14
Mean for group Z		186	166	+ 20
Mean for groups WX		166	165	+ 16
Mean for groups YZ		183	182	+ 19
Mean for groups WXYZ		174	173	+ 17

ference observed between these Italian and the American types of diet are not dependent on the dietary fat and must be explained on the basis of some other dietary difference.

Phospholopids and triglycerides in the serum

Pooled serum samples were prepared by mixing equal amounts from three men on each of the two days at the three-week point of each diet period. The three-man subgroups were formed by choosing from each 7-man group those highest in cholesterol for subgroup *a* and those lowest for subgroup *b* leaving out the man with the central cholesterol value. Subjects in each subgroup remained in those groups throughout the experiment. The pooled serum samples were analyzed for phos-

TABLE 4

The percentage of total calories supplied by saturated monoene and polyene fats in each diet, computed from table 2, and the predicted difference in serum cholesterol (Δ cholesterol) calculated from the equation Δ cholesterol = 2.68 ΔS — 1.23 ΔP

	Diet		Δ	Diet		Δ
	IL	AL	AL — IL	IM	AM	AM — IM
		Fat Cal. % of total Cal.				
Saturated, %	6.8	5.6	— 1.2	8.7	7.8	— 0.9
Monoene, %	7.7	8.9	+ 1.2	18.8	19.4	+ 0.6
Polyene, %	1.8	1.8	0	2.6	3.2	+ 0.6
		Predicted serum cholesterol effect				
2.68 Δ Saturated, *mg %*			— 3.2			— 2.4
— 1.23 Δ Polyene, *mg %*			0			— 0.7
Δ Cholesterol, *mg %*			— 3.2			— 3.1

TABLE 5

Serum lipid concentrations and differences caused by changing diets, mg per 100 ml

Group	Cholesterol		Phospholipids		Triglycerides	
	A diet	Δ	A diet	Δ	A diet	Δ
	AL	AL — IL	AL	AL — IL	AL	AL — IL
Wa	201	+ 20	206	+ 18	164	+ 23
Wb	171	+ 12	194	+ 18	122	+ 3
Xa	167	+ 25	171	+ 17	105	— 21
Xb	140	+ 14	164	+ 9	107	+ 1
Mean for groups WX	169.8	+ 17.8	183.8	+ 15.5	124.5	+ 1.5
	AM	AM — IM	AM	AM — IM	AM	AM — IM
Ya	220	+ 27	214	+ 22	144	+ 9
Yb	172	+ 12	180	+ 10	116	+ 18
Za	188	+ 25	193	+ 18	83	— 14
Zb	183	+ 17	192	+ 14	112	— 14
Mean for groups YZ	190.8	+ 20.2	194.8	+ 16.0	113.7	— 0.3
Mean for groups WXYZ	180.3	+ 19.0	189.3	+ 15.8	119.1	+ 0.6
Standard error of mean Δ		± 2.2		± 1.6		± 5.6

Each sample was a pool of serum from three men on two days at three weeks after the diet change. Subgroup *a* included the three men who averaged highest in serum cholesterol and subgroup *b* the lowest men, the 7th man being omitted. Cholesterol values were computed from the analyses of the individual samples. The diet designations above the data for each group are explained in table 3.

pholipids and for "total fatty acids and cholesterol" and triglyceride levels were computed. The data are given in table 5. The phospholipid values showed trends similar to the cholesterol values and the two lipids changed in parallel fashion in every subgroup. Subgroup *b* had a lower mean phospholipid value than subgroup *a* in every case. This indicates that there was a parallel relationship between these two serum lipid components discernible in the array of men as well as in the two dietary situations. It has previously been observed that serum phospholipid and cholesterol changes are highly correlated when diet fat is changed (Keys, Anderson and Grande, '58). The same correlation holds in the serum lipid responses reported here which are due to manipulation of dietary factors other than the fats.

The serum triglyceride values showed no relationship to the cholesterol trends and the mean triglyceride values were almost identical on the two types of diet. The between-days variability of the triglyceride values was about three times as large as that for cholesterol and for phos-

pholipids as is reflected in the values of standard error of mean △. In order to be significant at the 1% level of probability a change in triglyceride of 20 mg per 100 ml of serum would have been required.

DISCUSSION

The cholesterol lowering effect of the Italian type diet as compared to the American type diet is the chief point of interest. The former contained more fruits and vegetables of all kinds, especially dry legumes and green leafy vegetables, but was lower in sucrose, skim-milk solids and meat. The most likely cause of the difference between the effects of the diets would seem to be the difference in the nature of the carbohydrates or in some factors associated with carbohydrates such as cellulose, hemicelluloses or pectins. The diets producing lower serum cholesterol values were certainly higher in undigestible or difficultly digestible polysaccharides.

The possibility must be considered that differences in the proteins in the diet may have played a role. The American type diets provided slightly more total protein than the corresponding Italian types, 5 gm in the case of diets AL versus IL and 10 gm in the case of diets AM versus IM. Since all diets were fully adequate in proteins, it seems unlikely that this small difference, averaging only 7% of the total protein mean, could be influential. We have shown in similar controlled experiments at Hastings that variations in the diet protein level over the range of 10 to 20% of the total diet calories are without effect on the serum cholesterol level of man when other things are constant save for the isocaloric exchange of protein for carbohydrate (Keys and Anderson, '57). In those previous experiments both low and moderate fat diets were tested and doubling the protein intake by means of a casein supplement did not affect the serum cholesterol.

The diets differed in the source of about a fourth of the total proteins. The Italian type diets provided about 28 gm more protein from vegetables, fruits and legumes than the American type which made up the difference in proteins from meat and eggs. Since the amino acid composition of the proteins from these different sources differ, we note that the total amino acid composition of the American versus Italian types will differ to the extent that about one-fourth of the total protein was provided from these different sources.

From tables of data on the amino acid composition of the various foods used, we can make only a rough estimate as to the differences in the diets in regard to amino acids. The two types of diet shared a common supply of about 11 gm of protein nitrogen in the daily diet and differed in their source of about 4 gm. The largest difference in amino acids would seem to have been in the sulfur-containing amino acids, the Italian type diet providing something of the order of 3,000 mg of these daily while the American type diet provided about 10% more. Since this difference is far less than our previous experiments with different levels of proteins in the diet, we doubt that this difference in the present experiments is important.

An attempt to evaluate the effect of replacing bread by olive oil and soy protein in these experiments is possible although it involves comparing the cholesterol levels in different individuals. Good control was achieved in the items for which the experiment was primarily designed by giving both diets to each individual and by using opposite orders of treatment in the pairs of matched subgroups. This control is lacking in comparisons between individuals. When all the men were on the same diet in periods just before and just after those reported, the mean serum cholesterol level of groups YZ was 17 mg per 100 ml higher than that of groups WX. When this difference was used as a correction, diet IM was found to be associated with a mean cholesterol level 2 mg per 100 ml lower than diet IL. Similarly computed, diet AM gave a mean serum cholesterol level 1 mg % higher than diet AL. Apparently, the substitution of olive oil and soy protein for bread made no difference in serum cholesterol level. This is in agreement with our findings (Keys, Anderson and Grande, '58) that oleic acid is equal to "simple carbohydrates" (a mixture of one-third each of sucrose, potato starch and rice

starch) in its effect on serum cholesterol level.

A major reason for undertaking the experiments reported here was the apparent discrepancy between population averages for serum cholesterol observed in Naples, Italy, and in experiments in Minnesota. In Naples, middle-aged clerks and workingmen had average values of about 170 mg of cholesterol per 100 ml of serum (Keys, Fidanza and Keys, '55). Men of the same age at the Hastings State Hospital in Minnesota had an average of 223 mg per 100 ml on a diet in which 19% of the total calories were supplied by glycerides of saturated fatty acids and 4% from poly-unsaturated fatty acid glycerides (cf. Keys, Anderson and Grande, '57). The Neapolitan diet concerned averaged about 21% of total calories from fats but only rough estimates are available for the contribution of the several types of fatty acids. However, the true values could not be outside the range of 5 to 8% of total calories from glycerides of saturated fatty acids and 2 to 4% from polyenes. From the prediction equation derived from controlled dietary experiments (Keys, Anderson and Grande, '57, '59) we can estimate the expected average serum cholesterol difference associated with the differences in the dietary fats in Naples and in Hastings, other things being equal. Taking the best estimate of the averages for the two diets, we would have, following equation 2c (Keys, Anderson and Grande, '59):

$$\Delta \text{ cholesterol} = 2.68\ (19\text{-}7) - 1.23\ (4\text{-}2) = 29.7.$$

Taking the most extreme estimates to yield the largest possible expected serum cholesterol difference, we would have:

$$\Delta \text{ cholesterol} = 2.68\ (19\text{-}5) - 1.23\ (4\text{-}4) = 37.5.$$

But the observed difference between the two groups of serum was 53 mg cholesterol per 100 ml and these data indicate that some factor in addition to the dietary fat was operative to account for an average discrepancy of the order of 20 mg of cholesterol per 100 ml. The experiments reported here were devised in an effort to discover whether the difference between the contents of Neapolitan and Minnesota diets such as fruits and leafy vegetables, might be involved. The data obtained are in agreement with the hypothesis that these dietary items are responsible for much or all of the discrepancy observed earlier.

SUMMARY

Controlled experiments were carried out on men with diets differing in the proportions of carbohydrate calories supplied from various food sources. Diets IL and AL provided 13% of calories from protein and 16% from fats and were identical except for 17% of total calories from sucrose and milk carbohydrate in diet AL exchanged for equal calories in carbohydrates in fresh fruits, vegetables and legumes and for the small accompanying exchange of proteins involving 4% of calories. Seven men subsisted on diet AL, then changed to diet IL for 6 weeks each, while 7 matched men made the reverse change. Similarly, two matched groups of 7 men each subsisted in crossover experiments on diets IM and AM which corresponded to diets IL and AL except that total fats provided 31% of calories.

Serum cholesterol averages were 16 mg per 100 ml lower on the IL than on the AL diet and 19 mg per 100 ml lower on the IM than on the AM diet and these differences were statistically highly significant. Gas chromatograph analysis of the extracted mixed fats from the diets as fed indicated equality of IL versus AL and of IM versus AM diet in poly-unsaturated fats but the IL and IM diets provided slightly higher amounts of saturated fats than in comparison with the AL and AM diets. These fat differences would, of themselves, produce slight serum cholesterol differences (about 3 mg per 100 ml) in the direction opposite to that observed. It is concluded that sucrose and milk sugar tend to produce higher serum cholesterol values than equal calories of carbohydrates contained in fruits, leafy vegetables and legumes.

ACKNOWLEDGMENTS

These experiments were aided by the services of the staffs in Minneapolis: Mrs. Nedra Foster, Ethel Owens, Carol Barbknecht, Mrs. Charlene Honda, Dr. Paul Kupchs, and in Hastings: Mrs. Helen Williams, Mrs. Mary Jane Schweich and volunteers from the Brethren Service Com-

mittee: Gerald Rhodes, Gary McCann, Richard Roller and John Hall.

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Studies on the Toxicity of Indigofera endecaphylla

III. SEPARATION OF TOXIC FRACTIONS FROM SEED AND HERBAGE

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In an earlier paper, Hutton et al. ('58b) have assessed the degree and type of histological liver damage produced in mice by the toxic principle(s) in the seed of *Indigofera endecaphylla*. It was shown that this liver damage gave a reliable measure of toxicity.

A procedure for the extraction and separation of a toxic fraction from the seed and herbage of *I. endecaphylla* is described using the mouse as the test animal.

MATERIALS AND METHODS

As previously described, a strain of *I. endecaphylla* from Ceylon, designated C.P.I. 18557, was used in these experiments. A proprietary poultry meal was used as a control ration or for compounding rations containing fractions from the seed or from the dried herbage previously extracted with chloroform. The mice were offered 4 gm of the compounded meal per mouse per day. When testing the toxicity of the various fractions obtained, the routine adopted was for each mouse to receive a fraction (extract, residue or fraction of extract) from 2 gm of seed or dried herbage per day in the compounded meal. If the result appeared equivocal, the proportion of the fraction fed was increased to establish a more definite result. The diets were fed for 8 to 10 days, two to 5 mice receiving each sample under test. The toxicity of the extracts was also tested by means of subcutaneous or intraperitoneal injections. The procedure was to give three injections of 0.5 ml of the concentrated extract over a 36-hour period and the animals were killed some 12 hours after the last injection.

The C.S.I.R.O. strain of white mice weighing 20 to 30 gm were used. Water was provided ad libitum and the mice were weighed at regular intervals. Livers were removed from the mice as soon as possible after they died or were killed and then fixed in 10% formol saline. Paraffin sections were prepared and stained with hematoxylin and eosin. The histological changes seen in the livers were assessed and graded arbitrarily as slight, moderate or severe. No attempt was made to compare the degree of liver damage more accurately and only those changes easily recognized in routine hematoxylin and eosin sections were considered significant. These included generalized swelling of parenchymal liver cells, severe hydropic degeneration, fatty infiltration, cell necrosis and parenchymal collapse with inflammatory cell infiltration. As the object was to find the most toxic fractions, minor evidence of liver cell change, such as slight fatty infiltration, was not considered as significant.

Extraction procedure

Seed. The finely-ground seed was soaked in 0.1 N sodium hydroxide (500 ml/100 gm of seed) for 16 hours. Water was then added to aid dispersion, the mixture filtered and the pH of the filtrate adjusted to 6. The combined filtrate and washings at this stage made approximately 2.0 l per 100 gm of seed. The aqueous extract was made up to 50% with ethanol and centrifuged to remove protein and other substances insoluble in ethanol. Additional ethanol-insoluble material was removed by

Received for publication February 18, 1959.

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successive precipitations with ethanol (60, 70 and 80%). At each precipitation the volume of water was reduced by evaporation in a rotary film evaporator until at the final precipitation an extract from 100 gm of seed was concentrated in 50 ml of water.

Herbage. The finely ground herbage (leaf and stem) was given a preliminary extraction in a soxhlet apparatus with anhydrous chloroform for 4 hours. The chloroform-extracted residue from 200 gm of dried herbage was then boiled for two hours in 1.5 l of water and filtered. The insoluble material was boiled with a further 1.0 l of water, filtered and washed. The extract (combined filtrates and washings) was freed from protein as described in the preparation of the extract of the seed.

Absorption of toxic substance(s) on a cation-exchange resin. The plant extracts were then extracted with ether as a purification step before being passed through resin columns. Resins used included "Zeokarb 225 (H)," "225 (NH_4)" and "226 (NH_4)." An example of the absorption procedure follows. The resins were purified by treatment with 5 N HCl and converted to the required form by washing with 2 N acid or alkali. The resin size was 50 to 100 mesh/inch. An extract from 100 gm of seed was applied to a "225 (H)" column (2.5 × 17 cm) at a rate of 1 ml per minute. The column was washed with water (1.5 l) and the substances absorbed displaced with 2 N $NaOH_4$ (2.0 l). The solutions containing the substances absorbed and those not absorbed were concentrated in a film evaporator, adjusted to pH 6 and tested for toxicity.

RESULTS

Extractions from seed. Preliminary extractions with 80% ethanol and two aqueous solutions, mild acid and mild alkali, showed that a toxic extract could be prepared with all these solvents.

The mice fed the alkali extract were obviously ill after 7 days, and were therefore killed. Histological sections showed gross liver damage (fig. 1A). The mice fed the 80% ethanol extract and the acid extract were active when killed at 10 days. Sections of their livers showed appreciable evidence of liver damage, and so did the livers from the animals fed the residues from these two extractions. By contrast, the mice fed the residue from the alkali extract showed no toxicity or only very slight toxicity. Thus the alkali treatment appeared to result in a more complete extraction and all subsequent seed extracts were prepared in this way.

Ether and butanol extraction of the seed extract. Ether extracts prepared from the seed extract contained no measurable toxicity whether the extraction was carried out under acid or alkaline conditions, nor was a butanol extract of the neutral seed extract toxic. Prolonged ether extraction of a neutral seed extract (20 hours) still left a high degree of toxicity in the aqueous solution, with no apparent toxicity in the ether.

Absorption on ion-exchange resin columns. The toxic substance(s) was firmly held on "225 (H)" when the seed extract was passed through it. No toxicity could be detected in the washings from this column. The toxic substance(s) was weakly held on "225 (NH_4)" and "226 (NH_4)."

Figure 1B shows the liver from a mouse which received, over a period of 8 days, a fraction absorbed on "225 (H)" from 16 gm of seed. When the amount of seed extract in the "225 (H)" fraction was increased fourfold, (namely, 64 gm of seed), the mice became moribund in 5 days. The liver showed gross degeneration and necrosis. By contrast, a mouse fed the material not observed from 64 gm of seed on the "225 (H)" column was alive after 8 days and showed no histological evidence of liver damage when killed.

Toxic fractions from herbage. Previous feeding tests had indicated a similar toxic action of herbage and seed of *I. endecaphylla.* The green herbage is unpalatable to mice, but if it is dried and extracted with chloroform, then mice will eat the chloroform insoluble residue and develop liver damage (Hutton et al., '58b). Figure 1, C shows the gross liver damage in a mouse killed after 10 days of receiving a diet containing the residue from 20 gm of dried herbage. In this instance the chloroform extraction had been run for 4 hours. When the chloroform extraction was pro-

longed (e.g., 24 hours), there was occasionally some very slight evidence of toxicity in this extract. The toxin(s) could not be removed effectively from the herbage by any of the procedures used with the seed. However, fairly complete extraction was obtained by the hot-water procedure described. Aqueous extracts prepared from the chloroform-extracted residue of dried herbage produced toxic changes in the liver when fed or injected. Figure 1, D shows the histological appearance of a mouse liver from an animal which received three intraperitoneal injections over a period of 40 hours, each injection being of 0.5 ml of the extract (1-ml extract equivalent to 0.5 gm of dry herbage). When an extract from the herbage was passed through a "225 (H)" column, the fraction absorbed was shown to be toxic.

DISCUSSION

The results show that a potent toxic fraction can be obtained from an extract of the seed of *I. endecaphylla* by absorption on a cation-exchange resin. A similar fraction prepared from an extract of the dried herbage produced liver damage. The results from most of the experiments indicated that seed was more toxic than dried herbage.

From our results we can conclude that the toxin(s) is soluble in water but not soluble (or only sparingly so) in chloroform, ether or butanol. It is heat-stable (Hutton et al., '58a) and stable in weak acid or alkaline solution. It appears to act as a cation on an ion-exchange resin. The fact that the toxic principle(s) was less firmly held by the weak cation-exchange resin ("226") and by "225" in the ammonium form, than it was on the "225 (H)" resin, suggests that it was less basic than the basic amino acids.

We are unable to give quantitative values to the recovery yields in our fractionation procedure. However, when the toxicity of the cation fraction was compared with the toxicity produced by equivalent amounts of whole seed mixed with meal, it became obvious that we had lost an appreciable amount of the toxin during the separation, even though the discarded residues showed very little evidence of toxicity. It is known that mice fed 50% of seed and meal live only 6 to 13 days, while those fed 12.5% of seed live approximately 4 weeks (Hutton, '58). In investigation on the long term ingestion of low levels of *I. endecaphylla* seed,[3] it has been found that mice may not show any appreciable evidence of liver damage when given 2% of seed with meal for as long as 24 weeks. It seems reasonable to assume, therefore, that some of the residues may have contained small but significant amounts of toxin which the mice received in too small a dosage and/or for too short a period to develop obvious liver damage.

A drawback to this type of investigation is the considerable variation that occurs in the response of different animals to the same dose of material, which makes comparison between fractions difficult to assess quantitatively. The pattern of histological damage varied. With small doses, the initial damage appeared periportal in some mice (see fig. 1D); in others, it resulted in a generalized swelling of hepatocytes (fig. 1C), or led to changes which appeared most severe in the midzonal or centrolobular areas. No definite conclusion can be drawn as to the significance of these changes as yet since they may be influenced by dosage levels, time factors, individual variations or possibly change in the structure of the toxic material due to the extraction method used. More than one liver toxin may be present; a group of closely related compounds may be present and be separated to different degrees by the different methods employed.

The use of injection techniques allows a more sensitive and more rapid assessment than the feeding experiments. However, the use of intraperitoneal injections may be misleading unless small doses are given over an interval which allows the mice to survive and develop histological evidence of liver damage. Many mice die rapidly if injected intraperitoneally with large doses of extracts from innocuous plants. Subcutaneous injections are better, but, in general, require more material to achieve comparable results.

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[3] Windrum, G. M. 1958 Unpublished data.

We have tried the effect of feeding extracts of seed of *Indigofera suffruticosa* from Ceylon. Aqueous extracts of this seed failed to produce histological evidence of liver damage, and so did feeding 50% of the whole seed for 10 days. Prolonged feeding of 25% of feed for up to 24 weeks did not cause any significant changes in the mice livers. These experiments served as useful controls for the toxic seed.

Further fractionation of the cation fraction by displacement chromatography or by chromatography on cellulose columns and injection of the materials so obtained would be a logical step toward the isolation of the toxic substance(s).

SUMMARY

1. Methods are described for the extraction of the toxic principle(s) from the seed and herbage of *Indigofera endecaphylla*.

2. Potent toxic fractions can be prepared from the seed and herbage extracts by the use of cation-exchange resin columns.

3. The amount of toxic principle in the herbage appears to be less than in the seed.

ACKNOWLEDGMENTS

We wish to thank Dr. J. Griffiths Davies, Chief, C.S.I.R.O. Division of Tropical Pastures, for encouragement and advice throughout this project, and Dr. A. W. Pound, Director, Department of Pathology, Brisbane Hospital, for generous provision of facilities and for advice. Also we gratefully acknowledge the valuable technical assistance of L. B. Beall and R. Bullock of C.S.I.R.O. and the Brisbane Hospital, respectively.

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PLATE 1

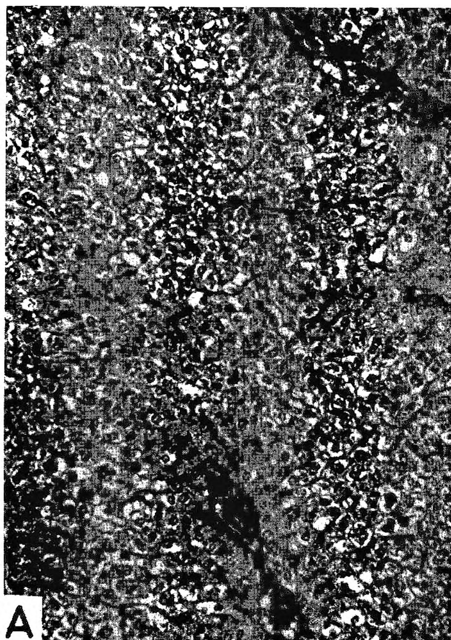
EXPLANATION OF FIGURES

1A Gross liver damage after 7 days' feeding of extract from 14 gm of seed. Liver, H & E. × 100.

1B Moderately severe liver damage after 8 days' feeding of fraction absorbed on "Zeokarb 225 (H)" column from 16 gm of seed. Liver, H & E. × 100.

1C Liver cell degeneration, necrosis and collapse after 10 days' feeding with 20 gm of chloroform-extracted dry herbage. Liver, H & E. × 100.

1D Liver damage after three intraperitoneal injections of extract from 1.5 gm of dried herbage. Liver, H & E. × 100.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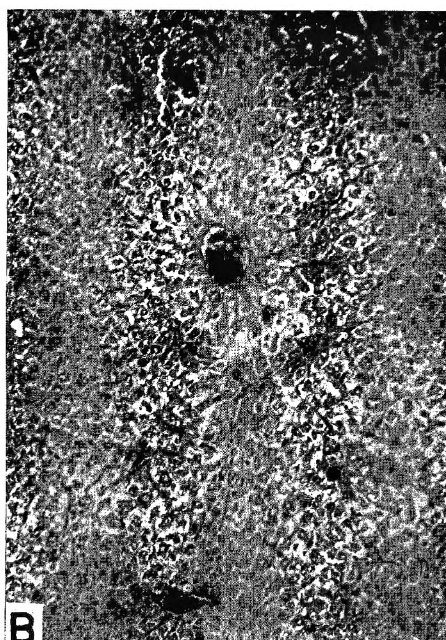
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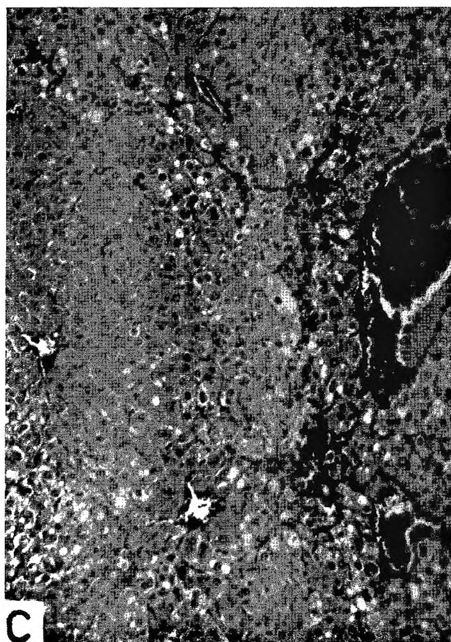
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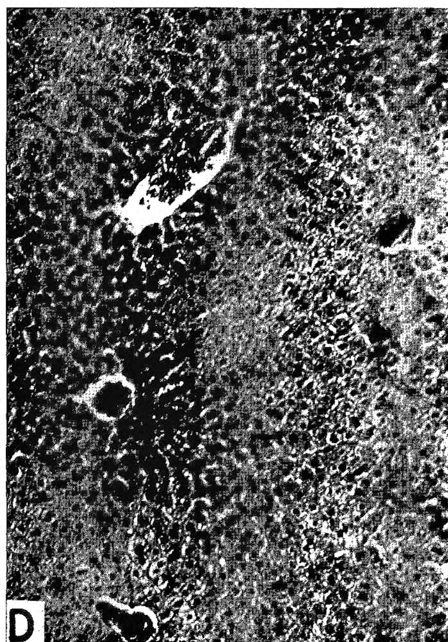
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Pathology of the Brain in Single and Mixed Deficiencies of Vitamins A and E in the Chick

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There is general agreement that the pathology of the central nervous system of the chick in vitamin E deficiency consists of large necrotic areas appearing usually in the cerebellum but occasionally in other areas of the brain (Pappenheimer and Goettsch, '31; Jungherr, '49). In the pathology of vitamin A deficiency, in contrast, the reports are conflicting. Wolbach ('54) stated that the symptomatology and pathology related to the central nervous system in the vitamin A-deficient chick is caused by compression of the central nervous system through the overgrowth of the bony structures surrounding it. Adamstone ('47) found small pinpoint lesions in the cerebellum and brainstem of vitamin A-deficient chicks. Rigdon ('52), however, found that the vitamin A-deficient duckling developed pyknotic, atrophic neurons in the spinal cord and vertebral ganglia.

In the course of studying the pathology of vitamin E deficiency in the chick, it became apparent that chicks exhibiting encephalomalacia frequently were simultaneously deficient in vitamin A. This raised the question as to the deficiency responsible for the observed pathology. For this reason, and because of the controversy in the literature about the effects of vitamin A deficiency on the central nervous system, the present experiments were undertaken to elucidate the pathology in uncomplicated deficiencies of vitamins A and E alone, and also as a combined deficiency.

EXPERIMENTAL

Day-old female New Hampshire chicks were fed purified diets deficient either in vitamin A or E. Basal diet C47 contained in per cent: purified soybean protein[2] 30; salt mix A (Briggs et al., '52) 6; DL-methionine 0.2; stripped lard[3] 4 to 25; and glucose to make 100. Adequate amounts of all B vitamins and vitamins D and K were added (Fox et al., '55). In addition, all diets contained 0.5 mg/kg of selenium as sodium selenite. This amount of selenium effectively prevents exudative diathesis but has no effect on the development of encephalomalacia. Diets were freshly prepared every two weeks. One jar of diet, sufficient for one week's feeding, was kept in the air-conditioned animal room (78°F) whereas the second jar was kept refrigerated until used.

When chicks exhibited definite symptoms of deficiency, as described below, but before they were moribund, they were killed with pentobarbital sodium and perfused through the heart with neutralized 10% formalin. After macroscopic examination, the brains were embedded in celloidin and cut serially at 20 micra. Every 20th section was stained with cresyl violet for cells and the sections were mounted in the usual manner.

RESULTS

Vitamin A status of vitamin E-deficient chicks. When diets prepared to be deficient in only vitamin E and containing 10 to 25% of lard were used, analyses of

Received for publication August 31, 1959.

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[2] Archer-Daniel Midland Company, Assay Protein.

[3] Distillation Products Industries.

the livers showed that the chicks were frequently simultaneously deficient in vitamin A. The vitamin A acetate in some experiments was given in the drinking water (Bieri, '57), in some it was added in ethanol directly to the diet at levels from 1.5 to 20 mg/kg, while in others 0.5 mg in water was given orally twice weekly. In all of these experiments, the majority of birds showing typical symptoms of encephalomalacia had no vitamin A in their livers when analyzed by the Carr-Price reaction. Furthermore, even when the vitamin A source and the vitamin E stress was 2% of fresh cod liver oil no vitamin A was detectable in the livers from chicks with encephalomalacia. Table 1 summarizes some of the analyses from various experiments. Although these chicks exhibited the characteristic symptoms of encephalomalacia, particularly the pedalling leg movements, apparently, biochemically, many of them may have had a deficiency of both vitamin A and E.

It was found that an adequate intake of vitamin A could be assured with the high lard diets by using a commercial stabilized dry vitamin A product.[4] When this was added at a level of 14 mg of vitamin A/kg of diet, the liver storage at 28 to 31 days of age amounted to 4 to 25 µg/gm in various series. The small amount of antioxidant in the stabilized product did not influence the onset of encephalomalacia.

These experiments vividly emphasized that chicks consuming vitamin E-free diets high in lard (10 to 25%) are extremely likely to be vitamin A-deficient also. Although this relationship between vitamins A and E had been known for a long time, a review of the literature suggests that in many studies of encephalomalacia the complication of vitamin A deficiency may have been present. In undertaking such studies it would appear imperative that analyses of the livers be performed routinely in order to assure investigators that vitamin A is adequate.

Vitamin A deficiency only. Chicks in this series were fed the purified diet containing 4% of lard with 100 mg of DL, α-tocopheryl acetate/kg. Analyses of livers for total tocopherols revealed 10 to 15 µg/gm. The ataxia of vitamin A deficiency appeared after 13 to 19 days and progressed rapidly during the next 18 to 48 hours. The picture was distinctly different from that of chicks deficient in vitamin E in that the "drunken" staggering and leg pedalling symptoms were absent.

Macroscopic examination of the brains and spinal cords of 9 chicks revealed no abnormalities. No herniation of the cerebellar tonsils was observed, pinching of spinal nerves, hydrocephalus nor other signs of increased intracranial pressure. Microscopically, 8 of the 9 brains showed only increased basophilia and pyknosis of scattered cells (fig. 1). These cells were especially common in the Purkinje cell layer of the cerebellum (fig. 2) and the inner layer of the optic tectum. One brain also had a few pinpoint lesions in the frontal lobes. This chick, in contrast to the others, was very emaciated and was

[4] Nopco Chemical Company.

TABLE 1

Liver storage of vitamin A in chicks fed purified vitamin E-free diets[1]

Vitamin A acetate added to diet	Amount and type of fat in diet	No. of chicks	Vitamin A in liver
mg/kg	%		*µg/gm*
1.5	lard, 25	3	0
5.0	lard, 25	5	0
10.0	lard, 25	6	2.6 ± 0.8
20.0	lard, 25	4	21.5 ± 3.8
14.0[2]	lard, 25	5	3.8 ± 1.0
0	cod liver oil, 1	1	0
0	cod liver oil, 2	4	0

[1] Chicks were killed between the 18th and 26th days when they exhibited classical symptoms of encephalomalacia.

[2] Vitamin A palmitate in the form of a dry, stabilized product.

killed more than 48 hours after the onset of symptoms.

Vitamin E deficiency only. The 13 chicks in this group received stabilized vitamin A in a diet containing 25% of lard and their livers were found to have adequate stores of vitamin A. The onset of symptoms occurred from 18 to 30 days of age and the typical picture was an inability of the chick to right itself even though it made frantic pedalling motions. Prostration, head retraction and emaciation eventually set in, ending in death.

Macroscopically, 4 of these brains exhibited dark red, soft areas in the cerebellum. Microscopically, large acellular necrotic areas were observed, usually in the cerebellum but occasionally in the optic lobes, frontal lobes and brain stem (fig. 3). No pinpoint lesions were seen, nor any vacuolar spaces surrounding vessels.

Combined deficiency of vitamins A and E. The 22 chicks in this category came from several series differing only in vitamin A intake. The diets contained 25% of lard, and as indicated above, vitamin A was added directly to the diet (1.5 and 3.0 mg/kg), put into the drinking water or given orally as described in the experimental section. No detectable vitamin A was found in the livers of these chicks. Analyses for vitamin E revealed a depleted level (Pollard and Bieri, '59). The symptoms, which appeared in 12 to 23 days, were characterized in some chicks by ataxia as seen in the group depleted of vitamin A only, whereas others were unable to stand. As the deficiency progressed, these birds became prostrate. Pedalling leg movements were observed in only a few of the chicks.

Macroscopically, the brains revealed no abnormalities. Microscopically, however, all brains exhibited pinpoint lesions to some extent (figs. 4 and 5). In sections showing only a few of these lesions (namely, in the early stage of deficiency), open areas (50 to 100 micra in diameter) always surrounded a blood vessel. The ground substances of the brain apparently had been pushed back from the blood vessel. In brains where large numbers of these pinpoint lesions were seen, a blood vessel could not always be detected in the center of the clear areas. These lesions were always seen in the frontal lobes and in a few instances they spread throughout the entire brain.

In addition to these findings, 8 chicks also had necrotic areas in their brains. These areas showed loss of architectural detail, fallout of all cellular elements and were sharply demarcated from the surrounding relatively normal brain. These large lesions, similar to those seen in chicks with encephalomalacia as described above, were all located in the cerebellum except for being in the frontal lobes in one chick.

DISCUSSION

The lesions found in the central nervous system of vitamin A deficient chicks were very limited. The pyknotic changes seen in these brains, however, are the same neuronal changes that Rigdon ('52) reported in the spinal cord and vertebral ganglia of the duckling. An interesting point about these vitamin A-deficiency changes is the lack of evidence indicating compression of the brain, increased cerebrospinal fluid pressure or bony overgrowth of the skull and vertebral column. This finding is contrary to that of Wolbach ('54) and Wolbach and Bessey ('41) and others who noted that the central nervous system was affected secondarily by compression from an overgrowth of the skull and vertebral column. A probable explanation for this disagreement may be found in the difference in time required to produce deficiency symptoms in different laboratories. The chicks in our study required less than 3 weeks to develop symptoms and undoubtedly were acutely deficient. This would be too short a time for a severe overgrowth of bone to take place.

The pinpoint lesions found in the combined deficiency of vitamins A and E are probably manifestations of edema from the small vessels of the brain. These lesions, though present in a somewhat different distribution, are similar to those described by Adamstone ('47) as being characteristic of the vitamin A-deficient chick. It appears highly probable that such lesions are a manifestation of a combined vitamin A and vitamin E deficiency. The observations of the difficulty of assuring an adequate intake of vitamin A when vitamin E-free diets with a high con-

tent of unsaturated fat, are used, emphasize the caution required in experimental nutrition studies.

The necrotic lesions found in pure vitamin E deficiency (encephalomalacia) and the large lesions in the combined deficiency of vitamins A and E, are similar to the characteristic lesions described by Jungherr ('49). It is significant, however, that many of these brains did not have gross pathology. Obviously, encephalomalacia cannot be diagnosed simply by the presence or absence of cranial hemorrhage.

SUMMARY

Day-old chicks were fed purified diets containing 4 to 25% of stripped lard. When the diets were made deficient in vitamin E, many chicks with encephalomalacia were found by chemical analyses to be simultaneously deficient in vitamin A. Special precautions had to be taken to assure an adequate intake of vitamin A. Histological comparisons of the central nervous systems revealed that brains from chicks deficient in vitamin A only had scattered pyknotic neurons, located most frequently in the optic tectum and Purkinje cell layer of the cerebellum. In vitamin E deficiency, large necrotic areas in the cerebellum and occasionally in other parts of the brain were seen. The combined deficiency of vitamins A and E resulted in many small acellular areas that were always found in the frontal lobes and occasionally in all parts of the brain.

ACKNOWLEDGMENT

The authors wish to thank Dr. Paul D. MacLean for providing the facilities for histological work.

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PLATE 1

EXPLANATION OF FIGURES

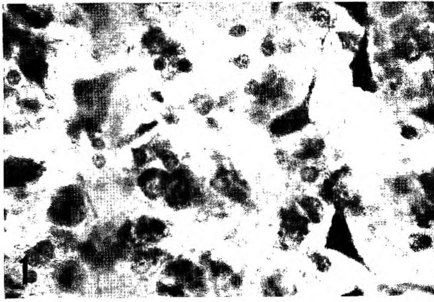
1 Darkly staining, pyknotic neurons are shown in the optic tectum of a vitamin A-deficient chick. Next to these cells are larger, lighter staining, more normal appearing neurons. × 400.

2 Two shrunken and pyknotic Purkinje cells can be seen in the cerebellum of a vitamin A-deficient chick. × 285.

3 Large, acellular necrotic areas in the frontal lobes of a vitamin E deficient chick. × 20.

4 Pinpoint lesions are spread diffusely through the frontal lobes of a chick deficient in both vitamins A and E. × 10.

5 A higher magnification of the pinpoint lesions in figure 4 showing that the substance of the brain is spread and vacuolated leaving relatively acellular areas. × 185.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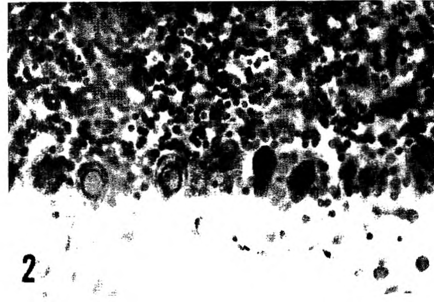
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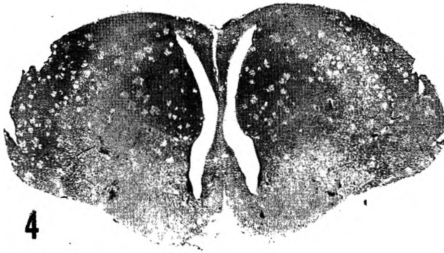
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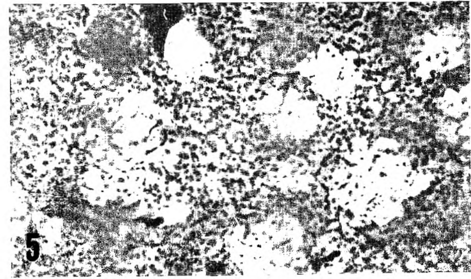
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The Vitamin C Status of Alcoholics[1]

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This study is designed to compare the vitamin C status of alcoholics to that of non-alcoholics, as assessed by the urinary excretion of oral, test doses of vitamin C. Jolliffe ('42) found vitamin deficiency diseases to be associated with alcoholism and Taverna ('54) found significantly low vitamin C levels in the blood of 30 alcoholic patients. The present study provides additional evidence to demonstrate that the vitamin C level of hospitalized alcoholics is substantially and significantly lower than that of non-alcoholics; the magnitude of this deficiency has been determined, establishing the basis for a rational program of nutritional therapy in these patients.

Low levels of vitamin C have also been observed to occur with significant frequency in the non-alcoholic population.

EXPERIMENTAL PROCEDURE

Vitamin C was administered to post-absorptive individuals in an oral, test dose of 500 mg. The bladder was emptied immediately prior to the administration of the test dose and the subsequent 4-hour excretion of urine collected, oxalic acid added to a concentration of 5% and preserved under refrigeration; the urine was analyzed for vitamin C by the method of Roe and Keuther ('43). The 4-hour period was chosen to facilitate the study; the shortness of the period produced no failures in urine collection.

The alcoholic population consisted of 85 male patients, 30 to 55 years old, who were tested on the day of admission to the Blue Hills Hospital of the Connecticut Commission on Alcoholism. The patients were either intoxicated or suffering from subsequent effects of having drunk liquor over three to 10 days. Intake of vitamin C preceding hospital admission was trivial. Ten of these patients were given 250 mg of vitamin C supplementation on the second through the 6th day and test doses of 500 mg on the first and 7th days of their hospital stay. Thirty patients were given 500 mg of supplementation daily and tested on the 7th day. Eight patients were given 1000 mg of supplementation daily and tested on the 7th day (supplementation was reduced to 500 mg on the 6th day in order to avoid possible residual vitamin C excretion on the test day; it had been determined that a 500 mg test dose could be excreted in 24 hours: a similar determination was not made for double this dose). The remaining 37 patients left the hospital against medical advice before a final test dose could be administered.

The control group consisted of 29 apparently healthy non-alcoholic subjects, 7 females and 22 males, aged 20 to 55 years, comprised of laboratory staff members and college undergraduates. Initially, 23 subjects were tested and then received daily vitamin C supplementation of 500 mg; 7 of these were tested on the 4th and 7th days, with 14 tested on the 7th day; two subjects did not complete the test. Another group of 6 non-alcoholic subjects was given 1000 mg of supplementation on the first, second and 5th days and 500 mg on the third, 4th, 6th and 7th days, and their vitamin C excretion was tested on the 4th and 7th days.

Neither alcoholic nor non-alcoholic individuals were allowed extraneous sources of high vitamin C content, such as citrus juices and vitamin tablets, on the days of testing.

Submitted for publication July 21, 1959.

[1] This research was supported by a grant from the Miles Laboratories, Inc., Elkhart, Indiana.

RESULTS

The urinary excretion of vitamin C is a function of the amount ingested and its level in the body. Johnson and Zilva ('34) found that a more or less constant level of urinary excretion was noted when the store of ascorbic acid was complete. Hafkesbring and Freeman ('52) also noted in saturation studies that the amount of vitamin C in the urine is in proportion to its blood plasma level and that at saturation the percentage excretion of a test dose of vitamin C is relatively constant. The level of vitamin C in the body thus determines that proportion of an administered dose which will be excreted. If the amount of vitamin C excreted equals the intake, less any loss by other routes, then the body is considered to be saturated with respect to this vitamin. Accordingly, the minimal daily requirement is that amount of vitamin C below which the percentage of a test dose excreted will diminish; the minimal daily requirement serves to maintain the tissue reserves of vitamin C at a maximal level (Storvick and Hauck, '42).

The results of this study are shown in table 1. From observations of the 21 non-alcoholic subjects (column C), receiving daily vitamin C supplementation of 500 mg for a week, we conclude that under our test conditions the mean excretion of vitamin C in 4 hours for an individual at saturation is $34.9 \pm 12.3\%$; any individual who excretes less than 10.3%, which represents two standard deviations below the mean, is by this criterion unsaturated or deficient in vitamin C. The P_D values in table 1 express the proportion of individuals in each group who were deficient on this basis.

Prior to supplementation (column A), alcoholic patients excreted a lower percentage of the test dose of vitamin C than non-alcoholic subjects (4.5% vs. 15.7%). Deficiency was observed in 85% of the alcoholic patients and 39% of the non-alcoholic subjects. The difference between these proportions (0.85 and 0.39) was significant at the 99% confidence level.

After a week of vitamin C supplementation of 250 mg daily, the excretion of 10 alcoholic patients (column B) had increased from 3.6 to 8.3%; 70% of these individuals still showed a deficiency at the end of the week. The 30 alcoholic patients who had received 500 mg of vitamin C supplementation daily (column C) increased their excretion from 4.8 to 22.4%; only 13% of these individuals were deficient at the end of the week. Neither the change from 3.6 to 8.3% nor the fall in the proportion deficient (column B) was significant, whereas that from 4.8 to 22.4%, as well as the fall in the proportion deficient (column C) was found to be highly significant. The results indicate that daily supplementation of 250 mg of vitamin C for a week is insufficient to correct the initial deficiency and that at least 500 mg daily is necessary.

In comparing the excretion of the 21 non-alcoholic subjects (column C) with that of the 30 alcoholic patients (column C), receiving the same supplementation, the difference in the mean excretions (34.9 vs. 22.4%) was found to be not significant, although the proportion (0.13) of deficient alcoholic patients differed significantly from zero. This indicates, we feel, that some importance should be attached to the lower mean level of excretion.

The data for the 8 alcoholic patients given 1000 mg of supplementation daily (column E) show the effect of larger amounts of supplementation. The mean of their excretion increased significantly from 2.6 to 29.8%, with only one patient deficient at the end of the week. Although a significant difference was not found between the mean of this group and that of the 30 alcoholic patients (column C), the fact that this mean is higher (29.8 vs. 22.4%) shows that amounts of supplementation larger than 500 mg tend to increase the mean excretion.

The increase from 15.4 to 34.9% in the mean excretion of the 21 non-alcoholic subjects (column C) was highly significant and shows that the non-alcoholic population was not initially at saturation. The value of 34.9% is only an approximation of the true saturation value even though it is not contradicted by the results noted for the 6 non-alcoholic subjects who received 1000 mg of supplementation (column E): no statistically significant difference is found between their mean excretions (45.0

TABLE 1

Urinary excretion of vitamin C

Day	Pre-supplementation (a)			Daily supplementation											
				250 mg (b)			500 mg (c)			500 mg (d)			1000 mg (e)		
	Mean excretion[1]	No. subjects	Proportion deficient[2]	Mean excretion	No. subjects	Proportion deficient	Mean excretion	No. subjects	Proportion deficient	Mean excretion	No. subjects	Proportion deficient	Mean excretion	No. subjects	Proportion deficient
	Alcoholic subjects														
1	4.5 ± 6.2[3]	85	0.85	3.6 ± 5.0	10	0.80	4.8 ± 7.0	30	0.83				2.6 ± 1.1	8	1.00
7				8.3 ± 7.4	10	0.70	22.4 ± 8.8	30	0.13				29.8 ± 20.1	8	0.13
	Non-alcoholic subjects														
1	15.7 ± 13.5	23	0.39				15.4 ± 13.0	21	0.38	19.5 ± 12.8	7	0.29			
4										30.4 ± 8.6	7	0.00	34.7 ± 10.1	6	0.00
7							34.9 ± 12.3	21	0.00	37.3 ± 11.8	7	0.00	45.0 ± 12.4	6	0.00

[1] Four-hour mean per cent excretion of 500 mg oral test dose of vitamin C.

[2] Percentage of individuals deficient in each group, excreting less than 10.3% of test dose.

[3] Standard deviation.

vs. 34.9%). There are also indications that those 6 non-alcoholic subjects who had received 1000 mg supplementation (column E) and the 7 non-alcoholic subjects who had received 500 mg of supplementation (column D) had reached a plateau of excretion, because no significant difference was found between mean excretions on the 4th and 7th days (45.0 vs. 34.7% and 37.3 vs. 30.4%). The fact that some increase was noted in each case, however, suggests that the true value for saturation is somewhat higher than 34.9%. The values for the 24-hour excretion of the 6 non-alcoholic subjects showed a mean of 95.9% of the 500 mg administered, which corresponds closely to the definition of saturation given; therefore, the 4-hour excretion value of 45.0% would appear to be closer to the true saturation value than that of 34.9%, even in the absence of data for the 24-hour period for the 21 subjects.

No evidence was noted that vitamin C excretion was affected by differences in age or sex.

DISCUSSION

In the absence of contrary evidence and criteria for optimal functioning of the organism, the vitamin C saturated state is assumed to be desirable. It appears best to err on the side of safety and set standards for non-deficiency that are unassailable: if more than 10% of a 500 mg test dose of ascorbic acid is excreted in 4 hours, then the individual tested is deemed non-deficient in C. Although the excretion of less than 10% is consonant with an apparent state of well-being, an objective test which is more sensitive than any set of clinical signs is desirable. By the standards of this study, then, a significant number of individuals in the non-alcoholic population were found to be deficient in vitamin C prior to supplementation; this condition was corrected by a vitamin C supplementation of 500 mg daily for 7 days, and, in fact, evidence indicates that it was corrected by the 4th day. J. Sigurjonsson ('51) found similar low results with 5 male subjects; using oral test doses of 10 mg/kg of body weight, he found that during the first 4 hours after an initial test dose, subjects excreted a mean of 0.6%; after 4 days of supplementation their mean excretion had increased to 20.6%. Based on 24-hour collection periods, Youmans et al. ('36) found comparable results.

The great variability (15.7 ± 13.5%) of the excretion data for the non-alcoholics, pre-supplementation, shows clearly that no specific value can be set as the normal excretion of vitamin C and this confirms the conclusion of Johnson and Zilva ('34) that "The urinary excretion of ascorbic acid under normal conditions of existence is variable."

The present results establish a reliable, and yet simple, method for determining the vitamin C status of individuals. The use of a 4-hour urine collection and an oral test dose of 500 mg lend themselves conveniently to practical application. Twenty-four-hour collection periods and intravenous test doses eliminate most sources of error and produce more accurate and complete results, as Wright, Lilienfeld and MacLenathan ('37) argue; however, the shorter collection period considerably reduces the probability of subject errors in carrying out the determinations.

Our data show clearly that the vitamin C status of the alcoholic upon hospital admission differs considerably from that of the non-alcoholic. This is clear from a comparison of the pre-supplementation tests alone. Supplementation of 250 mg of vitamin C for a week is inadequate to correct the deficiency in alcoholic patients; at least 500 mg daily is necessary to correct the initial deficiency, and because of the severity of the deficiency, even this amount does not raise the level in alcoholic patients to that of non-alcoholic subjects. An increase to 1000 mg daily raises the level somewhat. Because alcoholic patients tend to leave the hospital soon after admission, and considering the evidence from this study, we recommend that such individuals receive at least 500 mg of vitamin C supplementation daily for a week or more, after which possibly 150 mg daily might be used.

SUMMARY

Prior to supplementation, the percentage excretion of a 500 mg oral test dose of vitamin C was significantly lower for

85 alcoholic patients upon hospital admission than for 23 non-alcoholic subjects. Significantly more alcoholic patients than non-alcoholic were deficient in vitamin C, although a significant proportion (0.39) of non-alcoholics also were deficient.

The level of vitamin C excretion of the non-alcoholic subjects was raised to saturation value by administering 500 mg of vitamin C daily for a week; 34.9% excretion of an oral, test dose in 4 hours approximates the true saturation value.

Daily supplementation with 250 mg was inadequate to correct the initial deficiency in the alcoholic patients; at least 500 mg of vitamin C daily for a week is required in such patients before placing them upon a maintenance regime.

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PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA