ERRATUM

Nicolaysen, R., and R. Ragård 1961 Effect of various oils and fats on serum cholesterol in experimental hypercholesterolemic rats. J. Nutrition, 73: 299.

(Ms. error) On page 304, table 4 should have read as follows:

TABLE 4

Serum cholesterol values in long-term prophylactic test[1,2]

	No. of rats	Serum cholesterol		
		52 days	76 days	90 days
		mg/100 ml	*mg/100 ml*	*mg/100 ml*
Cod liver oil, 29.3% polyenoic fatty acids	5 male	109 ± 11[3]	131 ± 11	111 ± 5
	15 female	117 ± 4	114 ± 5	94 ± 5
Soybean oil, 61.1% polyenoic fatty acids	8 male	231 ± 25	129 ± 9	110 ± 5
	15 female	166 ± 17	136 ± 10	122 ± 6

[1] Diet: 10% of hydrogenated coconut oil, 1% of cholesterol, plus 2% of the test oil from the day the litter was born.

[2] According to food intake measurements in these female rats when 90 days old, the daily intake of polyenoic fatty acids was about 75 mg for the females fed cod liver oil and about 150 mg for those fed soybean oil.

[3] S.E. of the mean.

Effects of Feeding a Vitamin K-Deficient Ration Containing Irradiated Beef to Rats, Dogs and Cats[1]

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Treatment of foods with ionizing radiation could become an important food preservation technique. Poling et al. ('55) investigated the wholesomeness of ground beef irradiated with 1.9 megarads by feeding it to rats for two years. Their data indicated that irradiation did not significantly impair the nutritional value or wholesomeness of beef. A vitamin K deficiency was induced in rats, however, by feeding irradiated beef in a ration to which no vitamin K had been added (Metta et al., '59). Absence of hemorrhagenic compounds in irradiated beef when fed to rats was reported by Mameesh and Johnson ('59). The possibility that a hemorrhagic condition may occur in other species must be explored to determine whether irradiated beef can be considered acceptable for human consumption. The study of the occurrence of the hemorrhagic disease when a vitamin K-deficient ration containing irradiated beef is fed to rats, cats and dogs is the subject of this report.

MATERIALS AND METHODS

Ground raw beef and beef irradiated with 2.79 megarads[2] were supplied to us in sealed cans by the Department of the Army.[3] The irradiation procedure, storage conditions and chemical analyses have been reported (Reber et al., '60). The raw or irradiated beef was cooked in an autoclave for 25 minutes at 15 pounds pressure and was incorporated at a level of 35% of the total ration solids (table 1). The wheat flour was a commercial product. The flour, U.S.P. XIV salt mix, sodium chloride, and water were mixed to form a flaky material that was baked at 375°F for two hours. The baked material was ground in a hammer mill prior to mixing with the remainder of the ration ingredients.

Twelve male, weanling, Sprague-Dawley rats were fed the ration containing nonirradiated beef and 12 were fed the ration containing the 2.79-megarad irradiated beef. Rats were fed ad libitum, fresh food being offered three times a week. The experimental period was 17 weeks and rats were weighed once each week. Blood samples were obtained by heart puncture from 4 rats from each group at experimental weeks 1, 2, 3, 7, 9, 11, 13 and 16.

TABLE 1
Composition of experimental rations

	%
Wheat flour	48.0
Salt U.S.P. XIV	6.5
NaCl	0.5
Cellulose	3.0
Beef (untreated or irradiated)	35.0
Cod liver oil	1.5
Wheat germ oil	0.5
Vitamin mix[1]	5.0

[1] The vitamin mix contained: (in grams) thiamine·HCl, 0.250; riboflavin, 0.250; pyridoxine, 0.250; Ca pantothenate, 2.00; nicotinic acid, 1.00; *p*-aminobenzoic acid, 5.000; inositol, 10.000; choline chloride, 100.000; and Cerelose to total weight of 5000 gm.

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[1] These studies were supported in part by contract DA49-007-MD72800 with the Office of Surgeon General, Department of the Army. The opinions expressed in this publication are those of the authors and not necessarily those of the Department of Defense.

[2] Rad is defined as 100 ergs of radiant energy absorbed per gram of substance.

[3] Irradiation with gamma rays was carried out at the Atomic Energy Commission's Material Testing Reactor at Arco, Idaho, by the Phillips Petroleum Company.

Prothrombin times were determined on whole plasma according to the method of Quick ('38). Prothrombin times were not considered prolonged unless the clotting time was more than 20 seconds when rat blood samples were analyzed. The prothrombin time data were converted to their reciprocals and the average value plus or minus the standard deviation is presented.

The ration containing nonirradiated beef was fed to one mongrel male cat for 24 weeks. Three mongrel male cats were fed the ration containing the beef irradiated with 2.79 megarads for 40 weeks. The cats were weighed once each week. They were fed all they would eat in a one-hour period every day.

Blood samples were obtained from the jugular vein for prothrombin time analyses at the beginning of the experiment and at experimental weeks 4, 8, 12, 18, 24, 28, 33 and 40.

Two male beagle dogs were fed the ration containing nonirradiated beef for 24 weeks. Three male beagles were fed the ration containing the 2.79-megarad irradiated beef for 40 weeks. Four of the dogs were 11 weeks of age and one of those fed irradiated beef was 18 weeks of age at the beginning of the experiment. During the first 24 weeks of the experiment, all the dogs were offered the same amount of feed at each feeding. The amount of feed offered was limited so that all the feed offered was eaten. After experimental week 24, the dogs were offered all the feed they would eat during a one-hour period each day. Blood samples were obtained from the jugular vein for prothrombin time analyses at experimental weeks zero, 2, 6, 10, 14, 20, 26, 30, 35 and 40.

RESULTS AND DISCUSSION

Average weights of the rats and the number alive at the end of the experimental week listed are shown in table 2. Two rats fed the ration containing nonirradiated beef died as a result of accidents when blood samples were obtained. Nine of the 12 rats in the group fed the ration containing irradiated beef died because of hemorrhages.

No prolonged prothrombin times were observed in the blood of rats fed the ration containing nonirradiated beef. The mean reciprocal of the prothrombin time of 27 blood samples was 0.078 ± 0.011 seconds^{-1}. During the course of the experiment, 23 blood samples from rats fed the ration containing beef irradiated with 2.79 megarads were analyzed, and 5 of these showed prothrombin times longer than 20 seconds. The mean reciprocal of the prothrombin time was 0.068 ± 0.018 seconds^{-1}. The difference in the mean reciprocal of the prothrombin time values between rats fed the ration containing nonirradiated beef and irradiated beef was significant at the 1% level.

TABLE 2

Survival rate and average weight of rats fed nonirradiated and irradiated beef in a hemorrhagenic ration

Week	Nonirradiated		2.79-Megarad irradiated	
	No. alive	Av. weight	No. alive	Av. weight
		gm		*gm*
0	12	66	12	66
3	11	182	11	171
4	11	218	10	208
7	11	310	8	292
9	11	351	7	328
10	11	366	5	332
12	10	380	3	356
17	10	416	2	412

These data indicate that the ration containing the beef irradiated with 2.79 megarads was hemorrhagenic for male, Sprague-Dawley rats.

The initial body weight of the male cat fed the ration containing nonirradiated beef was 2340 gm, and it gained 510 gm during the 24-week period. The prothrombin time of the blood of the cat at the beginning of the experiment was 12.0 seconds (0.083 seconds^{-1}), and the mean reciprocal of the prothrombin time of 5 subsequent blood samples was 0.089 ± 0.012 seconds^{-1}.

Initial body weights of the male cats fed the ration containing irradiated beef were 2750, 3225, and 2925 gm and at the end of the 40-week experimental period they were 3100, 3550 and 4200 gm, respectively. The average weight gained was 650 gm. The mean reciprocal of the prothrombin times of the blood of the cats at the beginning of the experiment was 0.093 ± 0.009 seconds^{-1}. The mean reciprocal of the prothrombin time of 20 subsequent blood analyses was 0.089 ± 0.004 seconds^{-1}.

Feeding a ration containing irradiated beef which was hemorrhagenic for male Sprague-Dawley rats did not cause death, loss of weight or prolonged prothrombin times in male cats during a 40-week experimental period.

Two male beagles were fed the ration containing nonirradiated beef for 24 weeks. At the beginning of the experiment they weighed 1685 and 2425 gm, and at the end, 9300 and 10,440 gm, respectively. Average weight gain was 326 gm per week. The dogs were in good health throughout the experiment. The mean reciprocal of the prothrombin times of the blood obtained from the 5 dogs at the beginning of the experiment was 0.118 ± 0.017 seconds^{-1}. The mean reciprocal of the prothrombin time of 11 blood samples obtained during the 24-week period was 0.118 ± 0.020 seconds^{-1}.

The ration which proved to be hemorrhagenic when fed to male rats was fed to three beagles for 40 weeks. The dogs weighed 2850, 2474 and 4450 gm at the beginning of the experiment, and 17,250, 12,710 and 15,436 gm at the end of the experiment, respectively. Average weight gained per week was 297 gm during the 40-week period. The mean reciprocal of the prothrombin time of 21 blood samples obtained during the 40-week period was 0.131 ± 0.020 seconds^{-1}.

Feeding beef irradiated with 2.79 megarads in a ration which was hemorrhagenic for male rats did not cause death or prolonged prothrombin times in male beagles during a 40-week experimental period.

The amount of vitamin K in beef has been reported as 33 gamma per 100 gm (Metta et al., '60). The irradiation of beef with 2.8 megarads caused a 50% (Metta et al., '60) or 85% (Desrosier and Rosenstock, '60) loss of vitamin K. If beef contained 33 gamma of vitamin K per 100 gm and 50% was lost due to irradiation, a calculation indicates the animals fed the ration containing irradiated beef received 6 gamma of vitamin K per 100 gm of ration solids. The amount of vitamin K in the ration containing irradiated beef was adequate to prevent prolonged prothrombin times of the blood of cats and dogs, although it was inadequate for rats.

SUMMARY

Twelve male, weanling Sprague-Dawley rats, a male cat and two dogs were fed a ration containing nonirradiated beef. All the animals survived and there was no prolonged prothrombin time of the blood.

A ration containing beef irradiated with 2.79 megarads fed to 12 male, Sprague-Dawley rats caused the death of 75% of the rats because of hemorrhage. The mean reciprocal of the prothrombin time of the rats fed the ration containing irradiated beef was 0.068 ± 0.018 seconds^{-1}. When three male cats and three male dogs were fed a ration containing irradiated beef for 40 weeks, all gained weight and the prothrombin time of the blood remained normal. The amount of vitamin K in the ration was calculated to be 6 gamma per 100 gm of ration solids which was adequate to prevent prolonged prothrombin times of the blood of cats and dogs, although it was inadequate for Sprague-Dawley rats.

ACKNOWLEDGMENT

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Excretory Patterns and Bone Deposition of Zinc, Calcium and Magnesium in the Rat as Influenced by Zinc Deficiency, EDTA and Lactose

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Previous studies have shown that the presence of soy or of sesame proteins in purified diets increases the dietary zinc requirement of swine (Smith et al., '59), poultry (Moeller and Scott, '58; Morrison and Sarett, '58; O'Dell and Savage, '57; Lease et al., '60) and rats (Forbes and Yohe, '60) above that for animals fed casein or egg protein diets. The latter investigators have shown that the reason for the increased requirement is a lower absorption of the zinc contained in isolated soy protein compared with that contained in casein. Two methods, other than addition of zinc, have been used to prevent symptoms of zinc deficiency in animals fed these plant protein diets. Autoclaving (Smith et al., '60) was shown to improve zinc absorption and to thus reduce the need for supplementary zinc by swine fed a corn soybean meal diet. The need of poultry for supplementary zinc was reduced by autoclaving isolated soy protein diets (Kratzer et al., '59) and sesame meal diets (Lease et al., '60). The latter two papers also report that addition of the chelating agent, ethylenediaminetetraacetic acid (EDTA), to the diet apparently increased the availability of the zinc in these proteins. The newest development in this area is the demonstration that phytic acid, added to casein diets in a way to encourage development of a protein-phytate complex will intensify symptoms of zinc deficiency in chicks (O'Dell and Savage, '60) and in swine (Oberlease et al., '60; Plumlee et al., '60).

The current investigation was initiated in the fall of 1959 and had as its primary objective a study of the effects of EDTA and of lactose on pathways of excretion of zinc in normal and in zinc deficient animals. Lactose was chosen as an experimental variable because of its known stimulation of calcium absorption. Since there was also a chance that EDTA would influence calcium and magnesium excretion, these cations were also placed under investigation.

TABLE 1
Basal diet composition

	%
Isolated soy protein[1]	12
DL-Methionine	0.5
Corn oil	10
Glucose	66.2
Cellulose[2]	3
Vitamin-glucose mixture[3]	5
Vitamin A and D concentrate[4]	0.5
Mineral mixture[5]	2.8

[1] Archer-Daniel-Midlands Company, Cincinnati.
[2] Solka floc, Brown Company, Chicago.
[3] Forbes and Yohe ('60) but without chlortetracycline.
[4] Forbes and Yohe ('60).
[5] Forbes and Yohe ('60) but with 474 parts $CaHPO_4$ rather than 708.

PROCEDURE

Forty weanling male albino rats (Sprague-Dawley) were placed individually in stainless steel metabolism cages and were fed their respective rations for 6 weeks. During weeks 2, 4 and 6, collections of urine and of feces were made. During the entire feeding period the animals were given fresh feed daily and feed consumption was measured. Distilled water was available at all times. The cage unit was in a controlled environment room with a temperature of 27°C during the first and third two-week periods and 5°C during the middle period. The daily feed offered was limited to 8, 13 and 16 gm per rat during the three collection periods, so as to reduce spillage of feed.

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TABLE 2

Average daily grams of gain and feed intake of rats by two-week periods

Period	Temperature	Supplements							
		None		Zinc		Lactose		Zn and lactose	
		Gain	Feed	Gain	Feed	Gain	Feed	Gain	Feed
	°C								
		Diets without EDTA							
1	27°C	2.11	7	2.09	7	1.67	6	2.05	7
2	5°C	1.50	11	2.56	12	1.23	11	1.84	12
3	27°C	2.87	11	6.09	15	2.96	10	5.57	14
		Diets with EDTA							
1	27°C	2.19	7	2.21	7	1.80	7	2.04	7
2	5°C	2.20	12	2.26	12	1.96	12	2.26	12
3	27°C	5.01	14	6.47	16	3.66	12	5.27	14

The basal diet (table 1) was supplemented to provide EDTA (230 ppm of the disodium dihydrate salt), zinc (10 ppm as the carbonate), and lactose (25% as a replacement for an equal amount of glucose) in a complete factorial design. The basal diet contained 0.038% of Mg, 0.56% of Ca, 0.40% of P and 8.5 ppm of Zn. Five rats were given each diet. Rats and diets were randomly assigned within blocks.

At the end of the feeding period the rats were killed by stunning. Testes, seminal vesicles and prostate glands were removed, fixed in Bouin's solution and weighed in the fixed state. Femur bones were removed and analyzed for Ca, Mg and Zn. Balances of these cations were calculated from analysis of feed and excreta.

Zinc was determined by the method of Vallee and Gibson ('48) modified by use of a 0.01N HCl extraction to separate zinc from the mixture of dithizonates obtained particularly from feces samples. Calcium and magnesium were determined by the method of Malmstadt and Hadjiioannou ('59).

Data were treated by analysis of variance according to standard procedures.

RESULTS

Rats fed the low-zinc diets in this experiment exhibited only a few of the symptoms typical of zinc deficiency aside from a depressed feed intake and consequent reduction in weight gain. Most of these animals had a somewhat unkempt appearance; a few of them exhibited a moderate degree of hair loss.

The weight gain data in two-week periods are shown in table 2. Analysis of these data shows that zinc stimulated weight gain in each period, and that lactose reduced it. EDTA increased weight gain in periods 2 and 3 only. The addition of zinc had a negligible influence in the presence of lactose in period 1 and in presence of EDTA in periods 2 and 3. Feed intake within periods was linearly related to weight gain and treatment effects on weight gain may be entirely accounted for by the differences in feed intake. Gains of animals in period 2 equalled those in period 1 at a cost of 75% increase in feed intake.

The influence of treatment on zinc concentration and accumulation in femur ash

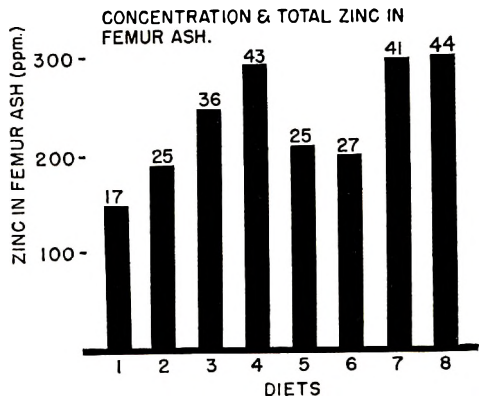

Fig. 1 Zinc concentration in femur ash (bars) and total micrograms of zinc per femur (numerals). Diets are, in order, 1 to 8: basal; basal + EDTA; basal + zinc; basal + zinc and EDTA; basal + lactose; basal + lactose and EDTA; basal + lactose and zinc; basal + lactose, EDTA and zinc.

TABLE 3

Weight of testes and final body weight

	Supplements							
	None		Zinc		Lactose		Zn and lactose	
	Testes	Body	Testes	Body	Testes	Body	Testes	Body
	% body weight	*gm*	*% body weight*	*gm*	*% body weight*	*gm*	*% body weight*	*gm*
Diets without EDTA	0.86	149	0.96	208	1.27	140	1.24	186
Diets with EDTA	1.13	184	1.10	212	1.17	162	1.09	191

is shown in figure 1. The major factor affecting the zinc concentration and deposition in the femur was dietary zinc, whose main effect was to raise the femur zinc from 188 to 285 ppm of the ash and from 23 to 41 μg per femur. A smaller but highly significant increase in femur Zn accompanied the inclusion of lactose in the diet. EDTA increased the zinc per femur and the zinc concentrations only in absence of the lactose effect. Concentrations of calcium (average = 38.8% ± 0.07) and of magnesium (average = 0.85% ± 0.14) in femur ash were not affected by any of the dietary treatments. Both zinc and EDTA increased the ash weight of the femurs 19 and 9%, respectively, but lactose had no effect on this measure of mineral utilization.

The weights of testes were positively correlated with those of seminal vesicles plus prostate. In order to eliminate effect of body size, data for testes were calculated as a percentage of body weight. Lactose increased testicular weight from 1.01 to 1.19% ($P < 0.01$), (table 3) an effect noticeable both in presence and absence of zinc, but not in the presence of EDTA since the latter increased testicular size of rats on the glucose diets.

In table 4 are shown the average data, by periods, obtained from the balance studies. The mean main effects of the various treatments are shown, together with their statistical significance in table 5. As a result of loss of some of the zinc data from 8 rats, only the data from the remaining 32 were treated statistically and included in the results of these balance studies.

Zinc balance. A study of these data shows that zinc absorption from the control diet was less in the first than in succeeding periods. In addition to a lesser absorption, there was a greater urinary zinc excretion in the early periods than in period 3. Hence zinc balance was least in period 1. Lactose did not, in the main, have an effect of zinc absorption or balance.

The effect of EDTA on zinc absorption was marked, and was greatest during the early periods. EDTA did not influence urinary zinc, hence the balance data parallel those for absorption. The main EDTA effect on zinc balance decreased from 16% in the first period to 4% in the third, and in the latter case was not of statistical significance.

The main effect of zinc supplementation was to decrease the percentage of zinc absorbed and retained, having little effect on urinary zinc excretion. In absolute terms, however, zinc retention was doubled by the zinc addition as shown in table 6. A zinc-EDTA interaction expressed itself significantly on percentage zinc balance, in periods 1 and 2, the EDTA effect being minimal in the presence of zinc as might be expected if the EDTA is effective for releasing "bound" zinc.

Calcium and magnesium balances. Percentage absorption and balance of both of these minerals was less in period 2 than in periods 1 and 3. Neither EDTA nor zinc had a consistent effect on absorption or balance of calcium or magnesium. Lactose increased urinary excretion of both calcium and magnesium. The increase in urinary calcium did not offset the increase in calcium absorption, so the net effect of lactose was to increase calcium balance. Magnesium absorption was only slightly increased by lactose, and because of the increased urinary excretion of magnesium, the balance of this element was decreased, although the decrease reached significance only in period 2. A zinc-EDTA interaction

TABLE 4

Average data, by periods, obtained during balance studies

Diet	No. rats	Feed, gm/ 7 days			Weight gains, gm/ 7 days			Zn absorbed			Zn balance			Ca absorbed			Ca balance			Mg absorbed			Mg balance		
Period		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
								%			%			%			%			%			%		
Basal	5	56	84	66	16	13	16	42	67	71	29	55	65	62	45	56	60	42	52	61	53	67	43	23	40
EDTA	4	56	90	92	17	14	28	73	84	82	67	77	77	63	52	46	61	49	44	67	61	74	43	27	45
Zinc	5	54	91	107	17	16	37	39	45	47	33	40	33	64	47	54	61	45	53	58	62	65	27	51	60
EDTA + Zn	4	56	91	112	18	15	42	49	55	54	36	50	50	68	53	56	66	51	54	65	60	67	31	23	32
Lactose	2	55	89	63	16	11	16	51	64	71	34	57	68	76	53	63	71	42	56	69	65	60	27	10	16
Lactose + EDTA	3	52	91	77	16	14	29	69	78	74	64	69	67	78	52	62	70	45	58	67	66	73	48	19	35
Lactose + Zn	4	56	91	101	17	12	35	51	50	50	46	46	46	76	50	59	70	43	54	67	63	80	39	19	40
Lactose + EDTA + Zn	5	55	91	95	17	14	28	48	60	49	41	54	45	72	50	62	67	46	58	69	64	82	26	17	41

TABLE 5

Mean main effects of zinc, EDTA and lactose on absorption, urinary excretion and balance of minerals (all figures indicate percentage of intake)

	Zinc absorbed			Urine zinc			Zinc balance			Calcium absorbed			Urine calcium		
Item	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Control group mean	42	67	71	13	12	6	29	55	65	62	45	56	2	3	4
EDTA	+14[1]	+12[1]	+5[2]	−2	−1	+1	+16[1]	+13[1]	+4	0	+3	−1	0	−2[2]	−1[2]
Zinc	−12[1]	−20[1]	−25[1]	−2	−3[1]	−2	−10[2]	−17[1]	−24[1]	0	−1	+1	0	−2[2]	−1
Lactose	+4	0	−3	−1	−1	−1	+5	+1	−2	+12[1]	+3	+9[1]	+4[1]	+6[1]	+3[1]

	Calcium balance			Mg absorbed			Urine Mg			Mg balance		
Item	1	2	3	1	2	3	1	2	3	1	2	3
Control group mean	60	42	52	61	53	67	18	30	27	43	23	40
EDTA	0	+5[2]	0	+3	+1	+6	0	+5	+7	+3	−4	−1
Zinc	0	+1	−2	−1	+1	+5	+8[2]	−8	−4	−9[1]	+8	+9
Lactose	+8[1]	−3	+6[1]	+5	+5[2]	+6	+6[1]	+20[1]	+17[1]	−1	−15[1]	−11

[1] P = 0.01.

[2] P = 0.05.

was found to be a result of zinc increasing magnesium balance in the absence of EDTA but not in its presence.

DISCUSSION

Data reported in this paper show clearly that EDTA does indeed increase the apparent absorption of zinc from a soy protein diet and by this mechanism alleviates a physiologic deficiency of zinc. The mechanism by which this is accomplished is not clear. O'Dell and Savage ('60), Oberlease et al. ('60) and Plumlee et al. ('60) have shown that the zinc in phytate-containing protein does not effectively protect animals against zinc deficiency. EDTA is a stronger chelating agent for the trace minerals than for the so-called macro-minerals. For example, the stability constants (-log dissociation constant) for metal-EDTA chelates are 19, 17, 16, 14, 11, 9 and 2 for Cu^{++}, Zn^{++}, Co^{++}, Mn^{++} or Fe^{++}, Ca^{++}, Mg^{++}, Na^{+}. These constants are pH-dependent, the optimum pH for complexing Mg^{++}, Ca^{++}, Fe^{++}, Zn^{++}, and Cu^{++} being 10, 8, 5, 4, and 3, respectively (West and Sykes). From this it may be inferred that even a low concentration of EDTA can complex trace metals at the acid pH of the stomach and perhaps the trace metal chelates will dissociate in the more alkaline medium of the intestine, thus permitting absorption of the released trace metals. This is doubtless too simple a system to represent accurately the biological situation since it is recognized that the chelating property of any ligand will depend not only on the factors listed above but also on rate of attainment of equilibrium, natural chelates present, and proportion of metal ions present.

It does seem clear that the zinc is not absorbed as the EDTA chelate. Studies on absorption of EDTA, using C^{14}-labeled material (Foreman et al., '53) have shown this to be extremely slight. Furthermore, the body seems unable to metabolize or store the Zn-EDTA chelate as shown by Millar et al. ('54) and Foreman ('60). The former investigators showed injected $CaNa_2$ EDTA to actually cause a rapid and large increase in urinary zinc excretion by rats, whereas the latter showed that, 24 hours after injection of inorganic zinc, 70% was retained in the body, whereas if $ZnNa_2$ EDTA were injected, only 12% of the dose was retained at 24 hours.

In an experiment conducted under similar conditions (Forbes and Yohe, '60) except that casein provided the major source of dietary zinc, no trend in absorption was found between the different collections, the average being 85 and 82% during weeks 2 and 6. It is possible that the increased absorption in the present instance, 42, 67 and 71% in weeks 2, 4 and 6, is a result of an enzymatic adaption which permits a greater release of zinc from the isolated soy protein.

Although none of the treatments in the current experiment affected the calcium or magnesium concentration of the bone ash, each treatment increased the concentration of zinc in the bone ash. Addition of zinc or EDTA would be expected to increase bone ash zinc since they caused a definite increase in total zinc balance and hence overcame a deficiency state. The effect of lactose is also explainable despite its lack of significant stimulation of zinc balance. Inspection of the data in table 4 shows that the direction of the lactose effect is toward an increased zinc balance in the absence but not in the presence of EDTA. That this difference is not statistically real, whereas the lactose effect on femur zinc is real, may be due to two factors. One is that the determination of bone zinc entails fewer complications and hence less experimental error than the determination of zinc balance. Another is that zinc balance measures all uses to which zinc is put in the body, whereas bone zinc measures only a fraction (28%) of the total body zinc. The observation that the effects of lactose and of EDTA on bone zinc concentration are not additive may be interpreted to indicate that the site of action of these materials is in the intestine, although it sheds no light on the mechanism.

The observation that balances of calcium and magnesium were less, 45 and 21% in the cold environment, than in the warm, 60 and 38% respectively, is logical in view of the fact that the diets were designed to provide adequate amounts of these minerals when consumed under "normal" conditions. While the rats

were in the cold, their energy intake was increased to a point which permitted gains nearly as great as obtained in the warm environment during the previous period. Hence although their body needs for these minerals were no greater, they were receiving more and hence would be expected to retain a smaller percentage. This observation fits well with that of McAleese and Forbes ('61) who demonstrated a lower percentage of magnesium required for bone magnesium deposition in rats kept in the cold than in those kept in a warm environment.

Observations of this experiment relative to the effects of lactose on calcium and magnesium utilization are in general agreement with the literature. The many experiments demonstrating a calcium-lactose interrelationship (see Duncan, '55) need little comment. The recent work on the mechanism of the lactose effect makes it possible to account in a general way for the fact that "lactose" both stimulates absorption and urinary excretion of the alkaline earth cations. The site of lactose action on calcium absorption now seems well pinpointed in the lower small intestine, perhaps extending to the colon (Lengemann, '59; Lengemann et al., '59; Vaughan and Filer, '60). It is also apparent from the work of these investigators that a wide variety of carbohydrates reaching the lower intestinal tract can stimulate alkaline earth absorption. Less attention has been devoted to the matter of increased urinary excretion of these minerals. The recent paper by Heggeness ('59) is interesting from this point of view. He shows that 60% of galactose in a rat diet does not affect calcium absorption but does cause a marked increase in urinary calcium and magnesium. It would thus appear that the increased urinary excretion of these minerals is associated with urinary excretion of galactose which surely occurs under the conditions existing (Mitchell et al., '37).

In the only previously reported study on the effect of lactose on magnesium balance (Outhouse et al., '38) it was noted that lactose increased magnesium absorption and urinary excretion but that the balance of magnesium was also increased. In the present investigation it was noted that the increased urinary excretion of magnesium was more than sufficient to offset the increased absorption so that the net effect of lactose was to decrease the magnesium balance. Differences in the experimental conditions, namely, in the earlier work the rats were vitamin D-deficient and the rations contained 830 rather than 500 ppm of magnesium, may account for the greater excretion of urinary magnesium with the resultant decreased balance observed as an effect of lactose in the present investigation.

SUMMARY

Forty weanling male albino rats were used in a 6-week growth and balance study of the effects of temperature, dietary lactose and supplements of ethylenediaminetetraacetic acid (EDTA) and of zinc on mineral balance of rats fed a basal zinc-deficient diet. Inclusion of 25% of lactose at the expense of glucose in the isolated soy protein diet was accompanied by decreased weight gain, increased absorption, urinary excretion and balance of calcium, increased urinary excretion and decreased balance of magnesium, and increased zinc concentration in the femur. Additions of zinc stimulated weight gain, total zinc absorbed and retained (although the percentage was decreased), and increased zinc concentration in the femur. Addition of EDTA increased weight gain, zinc absorption and retention, and concentration of zinc in the femur. At an environmental temperature of 5°C the percentage retentions of calcium and of magnesium were below those obtained at a temperature of 27°C. The effect of EDTA in stimulating zinc absorption decreased as a result of an increased zinc absorption from the basal diet as animals remained on experiment longer.

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All-Vegetable Protein Mixtures for Human Feeding

III. THE DEVELOPMENT OF INCAP VEGETABLE MIXTURE NINE[1]

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Protein deficiency is now widely recognized as a major nutritional problem among young children in most of the technically underdeveloped areas of the world (Anderson et al., '59; Béhar et al., '59) and in its severe form, kwashiorkor, it is responsible for the high mortality in the one- to 4-year age group in such regions (Béhar et al., '59; Pérez, '59). As emphasized in previous publications (Anderson et al., '59; Bressani et al., '59; Scrimshaw et al., '57; Squibb et al., '59), the development of suitable combination of vegetable proteins to supply the quantity and quality of protein required in the diet is an important approach to alleviating this situation in areas where milk and other food products of animal origin are costly or limited.

The successful development of a vegetable mixture for this purpose requires that its economic as well as its nutritive value be taken into consideration. INCAP Vegetable Mixture 8 (Bressani et al., '59; Scrimshaw et al., '57; Squibb et al., '59), containing 35% sesame flour, has excellent nutritive value, but is too costly for Central America because sesame seed is not produced in sufficiently large quantities and presents certain technical problems in harvesting the seed and producing the flour.

With the industrial production of relatively low gossypol and high-protein cottonseed flours suitable for human feeding and and the increase in cotton production in Central America, cottonseed flour is a more economical vegetable protein concentrate than sesame flour. It was decided, therefore, to try to develop a vegetable mixture eliminating sesame flour by increasing the proportion of cottonseed flour.

This paper presents studies carried out in chicks and rats to test cottonseed flour as a substitute for sesame flour, and to determine the most nutritive combinations of corn and cottonseed flour, which resulted in the development of INCAP Vegetable Mixture 9. Subsequent papers will describe further biological tests in chicks, rats, dogs and children.

MATERIALS AND METHODS

Biological tests involved both chicks and weanling white rats. A description of the materials used in the preparation of the diets tested has been given in previous publications. As described previously, the cottonseed flour[2] used was especially prepared for human consumption and had a higher protein quantity and quality and a lower gossypol content than that commonly used for animal feeding (Scrimshaw et al., '57). The sesame flour used in the baby chick rations contained 19% of fat, whereas in the rat diets, it was rendered fat-free by petroleum ether extraction.

Two series of biological trials were carried out with chicks. In series A, 4 experiments were conducted, adding cottonseed flour to replace sesame flour in INCAP Vegetable Mixture 8, keeping the protein percentage of the rations equal. In experiments 1 and 2 of series A, no lysine was added. In experiments 3 and 4 of this series, the level of lysine was kept constant at 1.00 gm per 100 gm of diet by decreas-

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[2] Pro-flo obtained from the Traders Oil Mill Company, Fort Worth, Texas.

ing the lysine supplement as the percentage of cottonseed was increased in the diet. The optimum combination of cottonseed flour with corn and sorghum grain was sought. In this series, the protein contribution of cottonseed flour varied from 75 to 90%, whereas the individual protein contribution of either corn or sorghum grain ranged from 10 to 25%.

One- or 4-day-old New Hampshire chicks of both sexes were distributed by weight and confined in battery brooders at controlled temperatures recommended for the age of the birds. Feed and water were provided ad libitum. Chicks were weighed individually every week for 28 to 35 days and the group diet consumption was reported weekly. In series A of the chick trials, 12 birds were allocated to each ration tested and 10 chicks per group were used in series B. The handling of the birds has been previously described. All diets used in the different chick feeding experiments were supplemented with a multivitamin solution (Bressani et al., '59).

Two series of trials were carried out with weanling rats of the Wistar strain of the INCAP colony. Series A was essentially the same as series A with baby chicks, except that there was no lysine supplementation; the sesame flour in the rations was oil-free and the level of protein was adjusted to 10% throughout. Series B of the rat trials with two separate experiments sought the best combination of corn and cottonseed protein in a design similar to that of the series B chick trial. The protein level in the diet was adjusted to 10%; corn protein varied from 100 to zero per cent and cottonseed zero to 100% in the

TABLE 1

Results of replacement of sesame flour by cottonseed flour in diets of chicks[1]

Ingredient	1	2	3	4	5	6	7	8	9	10
					Experiment 1					
Sesame flour[2]	26.25	22.50	18.75	15.00	11.25	7.50	3.75	—		
Cottonseed flour[3]	6.75	9.92	13.09	16.26	19.43	22.60	25.77	28.94		
Ground yellow corn	37.50	38.08	38.66	39.24	39.82	40.40	40.98	41.56		
Cornstarch	21.70	21.70	21.70	21.70	21.70	21.70	21.70	21.70		
Total	100	100	100	100	100	100	100	100		
Calculated protein, %	22.6	22.6	22.6	22.6	22.6	22.6	22.6	22.6		
No. of chicks, initial/final	12/12	12/10	12/11	12/12	12/12	12/12	12/11	12/12		
Initial weight, gm	50	50	50	50	50	50	50	50		
Final weight, gm	247	273	291	257	282	281	318	311		
Feed efficiency[4]	2.56	2.66	2.55	2.51	2.48	2.45	2.50	2.55		
					Experiment 2					
Sesame flour[2]	34.00	30.00	26.25	22.50	18.75	15.00	11.25	7.50	3.75	—
Cottonseed flour[3]	—	3.43	6.75	10.07	13.39	16.71	20.3	23.35	26.67	30.00
Ground yellow corn	37.50	37.50	37.50	37.50	37.50	37.50	37.50	37.50	37.50	37.50
Cornstarch	20.70	21.27	21.70	22.13	22.56	22.99	23.42	23.85	24.28	24.70
Total	100	100	100	100	100	100	100	100	100	100
Calculated protein, %	20.7	20.7	20.7	20.7	20.7	20.7	20.7	20.7	20.7	20.7
No. of chicks initial/final	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12
Initial weight, gm	48	48	48	48	48	48	48	48	48	48
Final weight, gm	235	225	253	260	283	244	312	336	342	337
Feed efficiency[4]	2.97	3.08	2.86	2.84	2.86	3.46	2.86	2.71	2.58	2.69

[1] All rations contained the following: (in per cent) kikuyu leaf meal, 2.25; Torula yeast (type 200, Lake State Yeast Corporation, Rhinelander, Wisconsin, courtesy of C. Bowman and Company, N. Y.), 2.25; mineral mix (Salmina, a commercial mineral mixture for chick feeding. Riverside Company, Guatemala), 3.0; cod liver oil (courtesy of Mead Johnson, Evansville, Indiana), 0.30; and vitamin solution (Bressani et al., '59), 1 ml/100 gm of diet.

[2] Sesame flour, 19% fat (*Sesamum orientale*).Obtained from the American Sesame Products Inc., Paris, Texas. Courtesy of Messrs. John H. Kraft and R. H. Anderson.

[3] Pro-flo, cottonseed flour, courtesy of UNICEF.

[4] Grams of feed consumed/grams of weight gained.

first experiment, and 5.3 to 30.3% for corn and 94.7 to 59.7% for cottonseed flour in the second experiment. Three animals of each sex were used per group and distributed by weight so that the average initial weights were the same among all the groups. The rats were placed in individual cages with raised screen bottoms, and received food and water ad libitum. Weight gains and food consumption records were kept for four 7-day periods. All diets were supplemented with a multivitamin solution (Manna et al., '53), and were analyzed for nitrogen content in order to determine the actual protein intake and calculate protein efficiency ratios.

The essential amino acid composition of the formula chosen was estimated using microbiological methods as described previously (Bressani et al., '58). The lysine, methionine and tryptophan content were also assayed from enzymatic hydrolyzates of the vegetable mixture (Bressani et al., '58) using microbiological methods.

RESULTS

The composition of the experimental diets, protein content, number of chicks per group, average initial and final weight and feed efficiencies of the first two experiments are shown in table 1. Replacement of the sesame flour by cottonseed flour tended to result in an increase in the final weight of the birds. Feed efficiencies remained constant in the first and appeared to improve slightly in the second experiment as the proportion of cottonseed flour in the rations increased. Analysis of variance showed these differences to be signifi-

TABLE 2

Effect of lysine supplementation on the replacement of sesame flour by cottonseed flour in diets of chicks[1,2]

Ingredient	1	2	3	4	5	6	7	8	9	10
					Experiment 3					
Sesame flour	28.00	24.00	20.00	16.00	12.00	8.00	4.00	—		
Cottonseed flour	7.20	10.70	14.20	17.70	21.10	24.60	28.10	31.60		
L-Lysine HCl	0.34	0.31	0.27	0.24	0.20	0.17	0.13	0.09		
Ground yellow corn	56.36	56.89	57.43	57.96	58.60	59.13	59.67	60.21		
Cornstarch	—	—	—	—	—	—	—	—		
Total	100	100	100	100	100	100	100	100		
Protein, calculated, %	24.1	24.1	24.1	24.1	24.1	24.1	24.1	24.1		
No. of chicks, initial/final	12/12	12/12	12/12	12/12	12/12	12/11	12/11	12/12		
Initial weight, gm	53	53	53	53	53	53	53	53		
Final weight, gm	540	567	564	538	574	578	532	499		
Feed efficiency[3]	1.83	1.93	1.89	1.93	1.97	2.04	2.00	2.07		
					Experiment 4					
Sesame flour	34.00	30.00	26.25	22.50	18.75	15.00	11.25	7.50	3.75	—
Cottonseed flour	—	3.43	6.75	10.07	13.39	16.71	20.07	23.35	26.67	30.00
L-Lysine HCl	0.34	0.30	0.27	0.23	0.19	0.16	0.12	0.08	0.04	—
Ground yellow corn	37.50	37.50	37.50	37.50	37.50	37.50	37.50	37.50	37.50	37.50
Cornstarch	20.36	20.97	21.43	21.90	22.37	22.83	23.32	23.77	24.24	24.70
Total	100	100	100	100	100	100	100	100	100	100
Protein, calculated, %	21.0	21.0	21.0	20.9	20.9	20.9	20.8	20.8	20.7	20.7
No. of chicks, initial/final	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12	12/12
Initial weight, gm	48	48	48	48	48	48	48	48	48	48
Final weight, gm	417	432	415	394	443	387	388	407	351	338
Feed efficiency[3]	2.14	2.11	2.17	2.27	2.11	2.38	2.36	2.34	2.48	2.65

[1] All rations in exp. 3 were supplemented with the following: (in per cent) kikuyu leaf meal, 2.40; Torula yeast, 2.40; mineral mix, 3.0; cod liver oil, 0.3; and vitamin solution (Bressani et al., '59), 1 ml/100 gm.

[2] All rations in exp. 4 contained the following percentages: kikuyu leaf meal, 2.25; Torula yeast, 2.25; mineral mix, 3.0; cod liver oil, 0.3; and vitamin solution (Bressani et al., '59), 1 ml/100 gm of diet.

[3] Grams of feed consumed/grams of weight gained.

TABLE 3

Effect of the replacement of sesame flour by cottonseed flour on the growth of young rats[1]

Ingredient	1	2	3	4	5	6	7	8	9	10
Sesame flour[2]	17.00	15.00	13.13	11.25	9.38	7.50	5.63	3.75	1.88	—
Cottonseed flour	—	1.72	3.38	5.04	6.70	8.36	10.02	11.68	13.38	15.00
Cornstarch	52.00	52.28	52.50	52.71	52.93	53.14	53.36	53.57	53.79	54.00
Average initial weight, gm	59	59	59	59	59	59	59	58	58	59
Average final weight, gm	163	159	157	156	156	162	152	149	156	154
Average weight gained,[3] gm	104	100	98	97	97	103	93	91	98	95
Feed efficiency[4]	4.40	4.47	4.55	4.43	4.53	4.39	4.49	4.66	4.43	4.42
Protein efficiency[5]	1.67	1.82	1.79	1.83	1.93	1.99	1.97	1.95	2.18	2.12

[1] All diets were supplemented with the following: (in per cent) ground yellow corn, 18.75; kikuyu leaf meal, 1.13; Torula yeast, 1.13; Hegsted ('41) mineral mix, 4.0; cod liver oil, 1.0; cottonseed oil, 5; and vitamin solution (Manna et al., '53), 3.0 ml.
[2] Fat free.
[3] Twenty-eight days.
[4] Grams of feed consumed/grams of weight gained.
[5] Average weight gained/average protein consumed.

TABLE 4

Value of combinations of corn or sorghum and cottonseed flour protein as determined in chicks[1]

Ingredient	1	2	3	4	1	2	3	4
Ground yellow corn	24.10	35.80	47.60	59.60	—	—	—	—
Ground sorghum grain	—	—	—	—	24.10	35.80	47.60	59.60
Cottonseed flour	36.40	34.30	32.30	30.30	36.40	34.30	32.30	30.30
Cornstarch	30.10	20.50	10.70	0.70	30.10	20.50	10.70	0.70
Percentage distribution of protein in diet								
from corn	10.3	15.3	20.3	25.4	10.3	15.3	20.3	25.4
from cottonseed	89.7	84.7	79.7	74.6	89.7	84.7	79.7	74.6
Protein, calculated, %	21.1	21.1	21.1	21.1	21.1	21.1	21.1	21.1
No. of chicks, initial/final	10/10	10/10	10/10	10/10	10/10	10/10	10/9	10/10
Average initial weight, gm	41	41	41	41	41	41	41	41
Average final weight, gm[2]	269	235	325	348	262	264	296	323
Feed efficiency[3]	2.19	2.08	1.92	2.00	2.04	2.08	2.01	2.09

[1] All diets were supplemented with the following: (in per cent) kikuyu leaf meal, 2.55; Torula yeast, type 200, 2.55; mineral mix, 4.0; cod liver oil, 0.30; and vitamin solution (Bressani et al., '59), 1 ml.
[2] Experimental period, 28 days.
[3] Grams of feed consumed/grams of weight gained.

cant at the 5% level in the first experiment and at the 1% level in the second.

Composition of the diets and results of the third and fourth experiments are shown in table 2. Growth of the birds and feed efficiency appeared to decrease as the concentration of cottonseed flour increased and the lysine addition decreased. Analysis of variance indicated that these differences are significant at the 5% level in experiment 3 and at the 1% level in experiment 4.

The effect of replacement of sesame flour by cottonseed flour in Vegetable Mixture 8, as carried out in the young rats, is shown in table 3 together with the composition of the experimental diets. The weight gains of the rats after 28 days on trial were not different from group to group. Feed efficiencies were also similar in all diets tested. Protein efficiencies, however, increased as cottonseed flour replaced more of the sesame flour in the diet. The all sesame-corn diet had a protein efficiency of 1.67 gm of weight gain per gm of protein consumed, while the all cottonseed flour-corn diet resulted in a protein efficiency of 2.12 gm.

The results of trials to determine the optimum combination of corn and cottonseed flour and of grain sorghum and cottonseed flour are shown in table 4. The table also shows the composition of the diets tested, the protein distribution of corn, sorghum and cottonseed flour, the number of chicks used and the protein content of the diets. The best growth and feed efficiencies were obtained when the cottonseed flour supplied from 75 to 80% of the protein of the ration, and corn from 20 to 25%. The same proportions were best when corn was replaced with grain sor-

TABLE 5

Value of combinations of corn and cottonseed flour protein as determined in young rats[1,2]

Ingredient	1	2	3	4	5	6	7	8
Ground yellow corn	—	6.15	12.05	17.90	23.80	29.80	35.72	87.46
Cottonseed flour	20.20	19.20	18.20	17.15	16.15	15.15	14.15	—
Cornstarch	67.26	62.11	57.21	52.41	47.51	42.51	37.59	—
Percentage distribution of protein in the diet								
from corn	0	5.3	10.3	15.3	20.3	25.4	30.3	100
from cottonseed	100	94.7	89.7	84.7	79.7	74.6	69.7	0
				Experiment 1				
Average initial weight,[3] gm		52	53	53	54	53	53	
Average final weight,[4] gm		138	137	147	137	137	150	
Average gain, gm		86	84	94	83	84	97	
Feed efficiency ratio[5]		4.20	4.23	4.10	4.39	4.21	4.16	
Protein efficiency ratio[6]		2.05	2.03	2.09	2.00	2.03	2.06	
				Experiment 2				
Average initial weight,[3] gm	54	54	54	54	54	54	54	54
Average final weight,[4] gm	151	146	141	157	141	139	140	95
Average weight gain, gm	97	91	87	103	87	85	86	41
Feed efficiency ratio[5]	4.38	4.48	4.47	4.30	4.66	4.69	4.92	9.24
Protein efficiency ratio[6]	2.03	1.91	1.99	2.05	1.87	1.82	1.78	1.22

[1] Protein content of diets: 10.2%.

[2] All diets were supplemented with the following percentages: kikuyu leaf meal, 1.27; Torula yeast, 1.27; Hegsted mineral mix, 4.00; cod liver oil, 1.00; cottonseed oil, 5.00; and vitamin solution (Manna et al., '53), 3 ml/100 gm of diet.

[3] Six rats per group, three males and three females.

[4] Experimental periods, 28 days.

[5] Grams of feed consumed/grams of weight gained.

[6] Average weight gained/average protein consumed.

ghum, although chick growth responded less than the whole ground corn.

In table 5 are shown the results of further studies of the optimum combination of corn and cottonseed flour tested in the growing rats. From the results of two experiments, the diet with corn contributing 15% and cottonseed flour 85% of the protein tended to be the best. This group had the highest growth and feed and protein efficiency. In general, all combinations tested resulted in better growth and feed and protein efficiency than either alone. The increase of corn protein above 15%, however, decreased both the protein and feed efficiency.

DISCUSSION

From the experimental results obtained in the chick trials, it can be concluded that cottonseed flour can satisfactorily replace sesame flour in chick rations. The sesame flour-based rations supplemented with lysine supported more rapid chick growth than the rations with cottonseed and no lysine. When lysine was not added, growth with the cottonseed flour-based ration was better than that with the sesame-based ration. These results can be explained partly by the higher content of lysine in cottonseed than in sesame flour (Orr et al., '57), although lysine is proportionally less available from the cottonseed flour (Altschul, '58). The better growth observed with the sesame flour ration supplemented with lysine was due to the addition of this amino acid. The cottonseed flour-based rations theoretically had the same amount of total lysine, but it was not entirely available to the chick. When the sesame flour was replaced by cottonseed flour and no lysine was added, however, the chick growth was better as the percentage of cottonseed flour in the diet increased because this constituent provided more lysine than the sesame flour.

The lower availability of lysine from cottonseed flour is due to operating conditions during the extraction of the oil that cause a reaction of the gossypol of the seed with the epsilon-amino group of lysine. This reaction binds the amino acid in such a way that it becomes biologically unavailable to the animal (Altschul, '58).

The results from the rat and chick trials designed to find the optimum combination of corn and cottonseed proteins indicate that the amino acids of cottonseed flour complement the proteins of corn which are deficient in the amino acids, lysine, tryptophan and isoleucine (Bressani et al., '58; Sauberlich et al., '53), resulting in a food with more nutritive value than either alone.[3]

The data from this group of experiments and calculations of the amino acids proportions in the combinations of corn and cottonseed flour led to the formulation of a provisional vegetable mixture with the following percentage composition: whole ground corn, 56; cottonseed flour, 38; Torula yeast, 3; and dehydrated leaf meal, 3. The results observed in the experiments discussed in this paper indicate that the proposed mixture would not lose its nutritive value if cheaper sorghum grain replaced all or part of the corn. This was based on the amino acid composition of sorghum as compared with corn (Bressani et al., '61) and from results with Vegetable Mixture 8 (Bressani et al., '59). Accordingly, INCAP Vegetable Mixture 9 was formulated with the following percentages: ground yellow corn, 28; ground sorghum grain, 28; cottonseed flour, 38; Torula yeast, 3; and dehydrated leaf meal, 3.

The essential amino acid content of the formula chosen is shown in table 6, as well as the pattern of amino acids in the FAO Provisional Reference Protein (FAO, '57). The comparison suggests that the order of limiting amino acids is methionine, with a score of 69%, followed by tryptophan with 74%, then lysine and isoleucine, slightly and equally limiting. The enzymatically obtained values for lysine and methionine are lower than the acid hydrolyzate values, whereas the tryptophan remained the same. If the enzymatic digest values are used for comparison, lysine becomes the first limiting amino acid followed by methionine and tryptophan. It is important for the further development and improvement of the nutritive value of the mixture to determine the extent to which

[3] Unpublished data by Bressani et al. reports similar results with lime-treated corn and cooked black beans, and with white rice and cooked black beans.

TABLE 6

Essential amino acid content of INCAP Vegetable Mixture 9

Amino acid			FAO standard	Score
	gm/100 gm	*gm/gm N*		
Arginine	2.34	0.531	—	—
Histidine	1.00	0.227	—	—
Isoleucine	1.12	0.254	0.270	94
Leucine	2.08	0.473	0.306	—
Lysine[1]	1.51	0.343	0.270	—
Lysine[2]	0.88	0.200	0.270	74
Methionine[1]	0.49	0.111	—	—
Methionine[2]	0.32	0.073	0.270	48[1]
Cystine[1]	0.081	0.018	—	34[2]
Cystine[2]	0.087	0.020	—	—
Alanine	1.52	0.345	0.180	—
Tyrosine	0.65	0.148	0.180	—
Threonine	0.87	0.198	0.180	—
Tryptophan[3]	0.24	0.055	0.090	61
Tryptophan[2]	0.26	0.059	0.090	66
Valine	1.14	0.259	0.270	96
Nitrogen, %	4.40	—	—	—

[1] Acid hydrolysis value. [2] Enzymatic hydrolysis value. [3] Alkaline hydrolysis value.

individual amino acids are biologically limiting.

SUMMARY

Biological trials with chicks and rats have shown that cottonseed flour can efficiently replace the sesame flour in Vegetable Mixture 8 made of the following percentage composition: lime-treated corn, 50; sesame flour, 35; cottonseed flour, 9; Torula yeast, 3; and kikuyu leaf meal, 3. Since good quality cottonseed flour contains more lysine than sesame flour, the replacement did not appear as effective when the level of lysine in the diets was adjusted to 1.00% by adding the free amino acid when needed.

Studies of corn and cottonseed flour combinations in both chicks and rats indicated that high protein nutritive value was obtained when corn contributed 15 to 25% and cottonseed flour 85 to 75% of the protein of the diet, and that sorghum could replace all or part of the corn in such a mixture without affecting the nutritive value.

The experiments presented resulted in the designation of a new formula for potential human consumption. Called INCAP Vegetable Mixture 9, it contained the following percentage composition: ground yellow corn, 28; ground sorghum grain, 28; cottonseed flour, 38; Torula yeast, 3; and dehydrated leaf meal, 3.

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All-Vegetable Protein Mixtures for Human Feeding

IV. BIOLOGICAL TESTING OF INCAP VEGETABLE MIXTURE NINE IN CHICKS[1,2]

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The initial biological trials with chicks and rats which led to the development of INCAP Vegetable Mixture 9, were presented in the previous publication (Bressani et al., '61). These studies demonstrated that cottonseed flour could replace sesame flour without reducing nutritive value and that the best results were obtained when corn contributed from 15 to 25% and cottonseed flour from 75 to 85% of the protein in the final vegetable mixture. To reduce the cost, the protein contribution from the cereal grain was divided equally between corn and sorghum grain since both have about the same protein value (Bressani et al., '59). These studies formed the basis for INCAP Vegetable Mixture 9 which has the following percentage composition: corn, 28; sorghum grain, 28; cottonseed flour, 38; dehydrated leaf meal, 3; and Torula yeast, 3. The previous publication has described the essential amino acid content of this mixture (Bressani et al., '61).

Two important aspects of the development of a vegetable mixture, its nutritive and economic value, have been discussed previously (Bressani et al., '61). This paper is concerned with still another step before such a mixture can be produced commercially. Any new type of vegetable mixture for human feeding must be biologically tested in at least two species of experimental animals before trials are carried out with human subjects (Waterlow et al., '57). Such experimentation is necessary not only to learn the nutritive value but also to determine any possible toxic effects. Biological trials of Vegetable Mixture 9 carried out with baby chicks are presented here.

MATERIALS AND METHODS

A description of all the ingredients of the experimental INCAP Vegetable Mixture 9 has already been given (Scrimshaw et al., '57; Squibb et al., '59; Bressani et al., '59). Vegetable Mixture 9 uses a lime-treated corn and sorghum, but as it is easier to feed raw grain to chicks, the formula actually employed was made with raw whole ground cereal grains. In addition to kikuyu (*Pennisetum clandestinum*) meal, other dehydrated leaf meals tested were ramie (*Boehmeria nivea*), quinamul (*Ipomonea sagittata*, lab.) and watercress (*Amarantus hybridus*). The leaf meals were prepared by drying fresh leaves for 48 hours with hot air at 80°C, then grinding in a Wiley Mill and storing at 4°C. The rice polishings, which were obtained from a rice mill in Guatemala City, had the following percentage composition: moisture, 14.4; protein, 12.5; crude fat, 18.0; crude fiber, 8.2; and ash content, 8.9.

All of the trials were carried out with New Hampshire baby chicks. The handling of the birds and length of the experimental period have been previously described (Bressani et al., '59, '61).

RESULTS

Corn and sorghum supplemented with cottonseed flour. Two trials were carried out that were similar except that by adding cornstarch, the protein content of the diets was adjusted to approximately 24% in the

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[2] INCAP Publication I-171.

first and 19% in the second. A lower protein level was used in the second trial to facilitate determination of the optimum complementation between the protein of the cereal grains and that of the cottonseed flour. In both trials the percentage of yellow corn and sorghum varied from 100 corn and 0 sorghum to 100 sorghum and 0 corn. The relative percentage of cottonseed flour was held equivalent to 38, the same ratio as found in Vegetable Mixture 9. All of the experimental diets were supplemented with the equivalent of 3% of kikuyu leaf meal and 3% of Torula yeast, amounts used in the formula for the vegetable mixture. The composition of the diets, number of chicks used per group and the results of the two trials are shown in table 1.

In the first trial, the different combinations of corn and sorghum had no significant effect on growth and feed efficiency and the values ranged from 78 to 82% of that found in the control group fed a commercial concentrate containing animal protein. In the second experiment, the 100% corn diet produced better growth due to higher food consumption. The feed efficiencies indicate that the 50–50 mixture of corn and sorghum is probably slightly superior although good results were obtained with all combinations.

Amino acid supplementation of Mixture 9 and substitution of the Torula yeast by rice polishings. From the amino acid composition of Vegetable Mixture 9 presented in a previous publication (Bressani et al., '61), it appeared that methionine might be the most limiting amino acid. Although lysine, as measured microbiologically, did not appear deficient in the mixture (Bressani et al., '61), it was thought to be limiting because of the reduced availability of this amino acid in cottonseed protein. Therefore, part of this experiment was done to study the effect of amino acid supplementation with methionine and lysine. Torula yeast is not yet available in the Central American region, and since this ingredient was added as a source of the B-vitamins, rice polishings were tested as a possible substitute.

The composition of the diets tested, the results of this trial and other pertinent data are shown in table 2. Growth and

TABLE 1

Growth response of chicks fed Vegetable Mixture 9 with varying combinations of corn and sorghum[1,2]

	Trial 1						Trial 2				
	1	2	3	4	5	6	1	2	3	4	5
Yellow ground corn	61.50	46.10	30.75	15.40	—	59.00	45.80	34.30	22.90	11.50	—
Ground sorgo grain	—	15.40	30.75	46.10	61.50	—	—	11.50	22.90	34.30	45.80
Cottonseed flour	30.40	30.40	30.40	30.40	30.40	—	22.60	22.60	22.60	22.60	22.60
Chick feed concentrate[3]	—	—	—	—	—	41.00	—	—	—	—	—
Protein, calculated, %	23.5	23.7	23.8	24.0	24.1	23.9	17.5	17.7	17.8	18.0	18.1
No. of chicks, initial/final	12/12	12/12	12/12	12/12	12/12	12/12	24/24	24/24	24/24	24/24	24/24
Average initial weight, gm	55	55	55	55	55	55	47	47	47	47	47
Average final weight, gm	479	464	460	466	479	587	299	264	238	228	224
Feed efficiency[4]	2.31	2.24	2.25	2.26	2.27	2.01	2.76	2.64	2.64	2.66	2.72

[1] All diets of trial 1, except no. 6, were supplemented with the following (in per cent): kikuyu leaf meal, 2.40; Torula yeast, 2.40; Salmina mineral salts (Bressani et al., '61), 3.00; cod liver oil, 0.3; and vitamin solution (Bressani et al., '59), 10 ml per 100 gm.

[2] All diets of trial 2 were supplemented with the following (in per cent): kikuyu leaf meal, 1.80; Torula yeast, 1.80; Salmina mineral salts, 3.00; cod liver oil, 0.30; and vitamin solution (Bressani et al., '59), 10 ml per 100 gm.

[3] Super Ace-Hi, Riverside Company, Guatemala.

[4] Grams of feed consumed per grams of weight gained.

TABLE 2

Effect of lysine and methionine supplementation of Vegetable Mixture 9 and replacement of Torula yeast by rice polishings on the growth and feed efficiency of chicks

Supplements to Vegetable Mixture no. 9[1]	Calculated protein	No. of chicks, initial/final	Average initial weight	Average final weight	Feed efficiency[3]
	%				
None	23.0	24/24	45	310	2.45
0.1% L-Lysine·HCl	23.0	12/12	45	486	2.13
0.2% L-Lysine·HCl	23.0	12/12	45	472	2.14
0.3% L-Lysine·HCl	23.0	12/12	45	494	2.07
0.2% DL-Methionine	23.0	12/12	45	357	2.28
0.3% DL-Methionine	23.0	12/12	45	361	2.26
0.4% DL-Methionine	23.0	12/12	45	361	2.44
0.2% L-Lysine·HCl + 0.3% DL-Methionine	23.0	12/12	45	490	2.04
Rice Polishings[2]	23.0	24/24	45	303	2.51

[1] To give 23.0 of protein, Vegetable Mixture 9 was made up of the following: (in per cent) ground yellow corn, 23.24; ground sorghum grain, 23.24; cottonseed flour, 31.54; kikuyu leaf meal, 2.49; Torula yeast, 2.49. All diets were further supplemented with 3.00% of Salmina mineral salts (Bressani et al., '61), 0.3% of cod liver oil, 10 ml of a vitamin solution (Bressani et al., '59), and 13.70% of cornstarch was used to adjust to 100%.

[2] In order to add rice polishings, Vegetable Mixture 9 was varied as follows: ground yellow corn, 26.32; ground sorghum grain, 26.32; cottonseed flour, 31.02; rice polishings, 7.52; kikuyu leaf meal, 2.82%. The ration was further supplemented with 3.0% of Salmina mineral salts (Bressani et al., '61), 0.3% of cod liver oil and 10 ml of a vitamin solution (Bressani et al., '59). To adjust to 100%, 27.0% of cornstarch was added.

[3] Grams of feed consumed per grams of weight gained.

feed efficiency improved slightly when 0.2% of DL-methionine was added, but greater amounts did not augment the nutritive value of the mixture. Similarly, the addition of 0.1% of L-lysine hydrochloride improved significantly both growth and feed utilization over the unsupplemented or methionine supplemented diets, but levels higher than 0.1% of lysine had no further effect. Addition of 0.2% of L-lysine hydrochloride and 0.3% of DL-methionine did not give better growth results than that obtained with lysine addition alone, although the amino acid mixture improved the feed efficiency over lysine-supplemented diets.

These results are of interest since the microbiological assays of the mixture for its amino acid content indicate that there is at least 1.0% lysine (Bressani et al., '61), an amount considered adequate for the requirements of the chick (N.R.C., '54). The response to lysine can be interpreted as due to a lack of availability of this amino acid from the cottonseed flour or other ingredient of the diet. The estimation of the lysine in the mixture using an enzymatic hydrolysis also indicated that lysine is not fully available (Bressani et al., '61). The substitution of rice polishings for Torula yeast and a small part of the cottonseed flour did not affect the nutritive value of the mixture. The growth of the chicks and the feed efficiency in the diet with Torula as compared to the one without Torula but with added rice polishings, were very similar.

Effect of lysine and methionine supplementation of Vegetable Mixture 9 and the effect of substituting rice or whole ground buckwheat for sorghum. Part of this trial duplicates the preceding one except that 0.1% of lysine and only 0.1% of methionine were added, alone or in combination. In this and in the previous vegetable mixture, Torula yeast was added mainly for its vitamin content. Torula yeast protein, however, is a relatively good lysine source (Orr et al., '57). In order to find out whether the lysine from this ingredient of the vegetable mixture was contributing towards its nutritive value as measured by chick growth, two diets were prepared without the yeast. One contained 0.1% of L-lysine hydrochloride supplement, an amount equivalent to that contributed by 3% of Torula yeast. The effect of replacing sorghum grain by rice or whole ground buckwheat was also studied in this trial.

TABLE 3

Effect of lysine and methionine addition and of the substitution of sorghum by buckwheat and rice in Mixture 9 on the growth and feed efficiency of chicks

Additions or changes in Vegetable Mixture 9[1]	Calculated protein	No. of chicks, initial/final	Average initial weight	Average final weight[2]	Feed efficiency[3]
	% of diet				
None	20.8	12/12	55	300	2.54
0.1% L-Lysine·HCl	20.9	12/12	55	382	2.45
0.1% DL-Methionine	20.9	12/12	55	324	2.51
0.1% L-Lysine·HCl plus 0.1% DL-methionine	21.0	12/12	55	416	2.19
Minus Torula yeast	19.7	12/12	55	250	2.79
Minus Torula yeast plus 0.1% L-Lysine·HCl	19.8	12/12	55	375	2.53
Plus ground buckwheat[4]	19.8	12/12	55	381	2.44
Plus ground white rice[5]	19.6	12/12	55	347	2.37

[1] The composition of the rations was as follows (in per cent): ground yellow corn, 21.0; cottonseed flour, 28.5; kikuyu leaf meal, 2.25; ground sorghum grain, 21.0; Torula yeast, 2.25; Salmina mineral salts (Bressani et al., '61), 3.00; cod liver oil, 0.30; and cornstarch to adjust to 100%. A vitamin supplement was also added (Bressani et al., '59).

[2] Experimental period, 35 days.

[3] Grams of food consumed per grams of weight gained.

[4] Ground buckwheat replaced totally the ground sorghum grain, see footnote 1.

[5] Ground white rice replaced totally the ground sorghum grain, see footnote 1.

The partial composition of the diets used in this experiment and the response of the chick to the several treatments are shown in table 3. The addition of 0.1% of L-lysine hydrochloride brought about a significant improvement in growth and in the efficiency of feed utilization compared with diets which did not receive the lysine supplement. The addition of 0.1% of DL-methionine did not significantly improve growth, either alone or in combination with the lysine, although feed efficiency improved. Omission of Torula yeast resulted in decreased growth and feed efficiency.

When the ration was supplemented with 0.1% of L-lysine hydrochloride and Torula yeast removed, improved growth and feed efficiency were observed similar to the group receiving both supplements together. Replacement of the grain sorghum by buckwheat or by white rice produced a highly nutritive vegetable mixture. The growth and feed efficiencies obtained were equal or slightly better than when the corn-sorghum-cottonseed-Torula yeast mixture was supplemented with 0.1% of L-lysine hydrochloride.

Effect of the substitution of dehydrated kikuyu leaf meal by other leaf meals and of Torula yeast by brewers' yeast. Five variations were studied in this experiment to determine the leaf meal contribution, if any, to the protein value of the mixture. One group was given Vegetable Mixture 9 with dehydrated and ground kikuyu grass, the leaf meal used in previous studies. Groups 2, 3 and 4 received dehydrated ramie, quinamul and watercress, respectively. A fifth group did not receive any leaf meal. In an additional group receiving kikuyu leaf meal, Torula yeast was entirely replaced by 2.25% of brewers' yeast.

The composition of the experimental diets and the results of the experiment are described in table 4. In general, all leaf meals were equally good with the exception of watercress which gave slightly lower growth and feed efficiency. None of the leaf meals appeared to improve the protein quality of the mixture. As compared with the effect of Torula yeast, brewers' yeast decreased both growth and feed efficiency due to a reduced food intake from the start of the trial.

Biological availability of lysine from Vegetable Mixture 9. This experiment consisted of comparing the growth of chicks fed a sesame meal ration supplemented with graded levels of lysine with that of birds receiving two levels of added Vegetable Mixture 9. From the value given in table 5, the amount of lysine in the vegetable mixture was calculated and com-

TABLE 4

Effect of the replacement of kikuyu leaf meal by other leaf meals and of Torula by brewers' yeast on the growth and feed efficiency of chicks

Variation in Vegetable Mixture 9[1]	Calculated protein	No. of chicks, initial/final	Average initial weight	Average final weight[2]	Feed efficiency[3]
	% in diet		*gm*	*gm*	
Plus kikuyu leaf meal[4a]	20.8	12/12	50	344	2.73
Plus ramie leaf meal[4b]	20.8	12/12	50	356	2.43
Plus quinamul leaf meal[4c]	20.8	12/12	50	318	2.61
Plus watercress leaf meal[4d]	21.1	12/12	50	303	2.92
No leaf meal added[5]	20.8	12/12	50	331	2.56
Plus kikuyu leaf meal, brewers' yeast substituted for Torula yeast[6]	20.7	12/12	50	259	2.90

[1] The composition of the basal ration in percentage was as follows: ground yellow corn, 21.0; ground sorghum grain, 21.0; cottonseed flour, 28.50; Torula yeast, 2.25; leaf meal, 2.25; Salmina mineral salts, 3.00; cod liver oil, 0.30; and enough cornstarch to adjust to 100%. All diets were supplemented with a vitamin solution (Bressani et al., '59).

[2] Experimental period, 35 days.

[3] Grams of feed consumed/grams of weight gained.

[4] 2.25% leaf meal was added each replacing kikuyu leaf meal of the mixture: (a) *Pennisetum clandestinum*, (b) *Boehmeria nivea*, (c) *Ipomonea sagittata*, (d) *Amaranthus hybridus*.

[5] Ground yellow corn was increased from 21.00 to 23.25%.

[6] 2.25% of brewers' yeast replaced the same amount of Torula yeast.

TABLE 5

Biological availability of lysine from Vegetable Mixture 9 for the chick[1]

Ingredient	1	2	3	4	5	6
Ground yellow corn	57.20	56.08	56.95	56.82	50.90	45.70
Sesame meal[2]	39.50	39.50	39.50	39.50	30.00	21.00
L-Lysine·HCl	—	00.125	00.250	00.375	—	—
Vegetable Mixture 9[3]	—	—	—	—	15.80	30.00
L-Lysine content, gm/100 gm	00.684	00.784	00.884	00.983	00.783	00.869
No. of chicks, initial/final	12/11	12/12	12/11	12/12	12/12	12/12
Average initial weight, gm	43	43	43	43	43	43
Average final weight,[4] gm	136	210	280	296	141	168
Feed efficiency[5]	3.21	2.43	2.34	2.04	2.91	2.68

[1] All diets were supplemented with 3.0% of Salmina mineral mixture, 0.30% of cod liver oil and 10 ml of a vitamin solution (Bressani et al., '59).

[2] The sesame meal contained 1.30% of lysine.

[3] Vegetable Mixture 9: ground yellow corn, 28.0; sorghum, 28.0; cottonseed, 38.0; kikuyu leaf meal, 3.0; Torula yeast, 3.0%.

[4] Experimental periods, 28 days.

[5] Grams of food consumed per grams of weight gained.

pared with microbiologically determined lysine following acid hydrolysis. The biological availability of lysine in Vegetable Mixture 9 estimated by this method averaged 83%.

Effect of the substitution of corn-sorghum in Vegetable Mixture 9 by other cereal grains. There are regions where corn or sorghum are not plentiful while other cereal grains predominate as important staples. The practical use of a vegetable mixture such as the one being discussed would increase if other cereal grains could be used without altering the nutritive value of the mixture. In this experiment, therefore, corn and sorghum were replaced by whole ground corn, barley, rice, oats, whole ground wheat and wheat flour. The composition of the diets and the growth response of the chicks are given in table 6. It can be seen that, in general, the growth of the chicks was satisfactory. Barley and rice induced the best growth, followed by corn, oats, whole wheat and wheat flour.

TABLE 6

Effect of the replacement of corn-sorghum in Vegetable Mixture 9 by other cereal grains on the growth and feed efficiency of chicks

Variation in formula of Vegetable Mixture 9[1]	Protein content of diet	No. of chicks, initial/final	Average initial weight	Average final weight[2]	Feed efficiency[3]
	%		gm	gm	
Plus wheat flour[4]	25.3	17/17	45	357	2.32
Plus ground yellow corn[4a]	24.5	17/17	45	399	2.17
Plus ground barley[4b]	25.9	17/17	45	420	2.27
Plus ground white rice[4c]	24.7	17/17	45	426	2.07
Plus ground oats[4d]	24.7	17/17	45	383	2.30
Plus ground whole wheat[4e]	25.8	17/16	45	380	2.41

[1] The basal diet consisted of the following (in per cent): cottonseed flour, 33.16; Torula yeast, 2.62; kikuyu leaf meal, 2.62; Salmina mineral salts, 4.0; cod liver oil, 0.30; and 48.87 of each cereal grain tested. Cornstarch was added to adjust to 100%. All diets were supplemented with a vitamin solution (Bressani et al., '59).

[2] Experimental periods, 35 days.

[3] Grams of food consumed/grams of weight gained.

[4–4e] The protein content of the cereal grains was: 12.0, 7.7, 10.4, 9.3, 11.8 and 12.6% for wheat flour, corn, barley, rice, oats and whole wheat, respectively.

The feed efficiency did not follow the growth response obtained, since the ration with rice showed a high value followed by corn, barley, wheat flour, oats and whole wheat.

DISCUSSION

Prerequisites of a vegetable mixture for human feeding are adequate nutritive value and freedom from possible toxic effects. The results of the series of experiments carried out and presented in this paper indicate that Vegetable Mixture 9 has good nutritive value and is free from toxic effects in the chick. The evidence also indicated that any proportion of corn and sorghum was adequate for practical use since no major change in nutritive value was apparent. Other cereal grains can be used satisfactorily, particularly rice. This finding is of importance because the economic aspects of the mixture must be taken into consideration as well. In regions where rice is a more important staple food than corn, its use might make the mixture more economical and acceptable.

There is apparently a lack of agreement between the lysine value as reported in a previous publication (Bressani et al., '61) and the results of lysine supplementation presented in this paper. The difference is explained partly by the fact that the lysine in Vegetable Mixture 9 is only 83% available to the chick. This lack of availability is apparently due to the fact that some of the lysine becomes bound with gossypol during the processing of cottonseed (Altschul, '58). The availability of lysine in the cottonseed flour depends on the conditions of processing; meal which is overheated has too low a protein value for use in preparing the vegetable mixture.

Although Torula yeast contributes toward the final nutritive value of the vegetable mixture, the results support the findings of Tsien et al. ('57) that not all the lysine in Torula yeast is completely available. The addition of 0.1% of lysine without Torula yeast had the same growth results as yeast added in the presence of lysine. It is also possible that the level of lysine in the mixture was greater than needed so that other amino acids were limiting; to get the full benefit of amino acid additions to a deficient protein, only the quantity that will balance the second most limiting amino acid is necessary (Rosenberg, '59). Of special importance is the fact that ordinary brewers' yeast cannot replace Torula yeast. Further processing of brewers' yeast, however, can render it free of the bitter factor which lowers food consumption (Sure, '58).

The results of the use of different leaf meals indicate that they do not contribute to the protein value of the mixture, although they are good sources of provitamin A. It is possible and advisable to exclude the 3% of leaf meal from the

mixture and add synthetic vitamin A instead as the carotene content of leaf meals is easily lost in preparation.

In general, the results presented in this paper indicate that Vegetable Mixture 9 has a high nutritive value and should give excellent results in preventing protein malnutrition in human beings, providing a good grade of cottonseed flour is used. No toxic effect of gossypol was observed in any of the trials. Advances in cottonseed technology and selection of varieties with seeds containing little or no gossypol (Mattson et al., '60) will undoubtedly facilitate the preparation of Vegetable Mixture 9. The results clearly demonstrate that proper combination of vegetable proteins can yield mixtures of good quality and low cost.

SUMMARY

A series of experiments carried out to test the nutritive value and lack of toxicity of INCAP Vegetable Mixture 9, was discussed. The mixture contains in per cent: corn, 28; sorghum grain, 28; cottonseed flour, 38; Torula yeast, 3; and leaf meal, 3. Any corn-sorghum proportion can be used without altering the nutritive value of the mixture, and other cereal grains and carbohydrate-rich seeds can replace the corn and sorghum. Among these, buckwheat and rice were found to be particularly good substitutes to improve chick growth and feed utilization. Other cereal grains or grain products tested were barley, oats, whole wheat and wheat flour.

The Torula yeast contributes significantly toward the protein value of the mixture for chicks. When brewers' yeast was substituted, the bitter taste resulted in poor growth and feed efficiency due to a lowered feed consumption. The several carotene-rich leaf meals tested did not contribute to the protein quality of the mixture. Because of this finding and the technical difficulties encountered in the preparation of good quality leaf meals, synthetic vitamin A preparations are recommended as substitutes.

The addition of 0.1% of L-lysine hydrochloride to the mixture brought about improved growth response in chicks suggesting that the mixture was limiting in lysine, although according to calculations based on microbiological amino acid analyses, lysine was not a deficient amino acid. The response to lysine supplementation was explained by the finding that lysine availability for the chick in the cottonseed flour used was only about 83%. The addition of 0.1% of methionine did not improve growth or feed efficiency either alone or in combination with lysine.

It was concluded that Vegetable Mixture 9 is of high nutritional value and of potential value in human feeding.

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Variations in Linoleic Acid Content of Dietary Fat in Relation to Metabolism of Fat, Nitrogen and Minerals, and to Changes in Blood Lipids[1]

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The results of numerous studies (Kinsell et al., '56; Ahrens et al., '57; McCann et al., '59; Keys et al., '59) have indicated that the feeding of fats having high linoleic acid content affects blood lipid levels in man. In many of the data accumulated to date the effect on serum cholesterol levels has been of major interest. Okey et al. ('60) studied the effect on other plasma lipids, including the distribution of saturated and unsaturated fatty acids, when safflower oil or coconut oil was substituted for an estimated 80% of the dietary fat in the usual prison diets of healthy 50-year-old men. Few data are available, however, concerning the effect of dietary linoleic acid on the metabolism of nutrients other than fat. A metabolic study was undertaken, therefore, to determine whether there were any demonstrable changes in fat excretion, in nitrogen and mineral retention, and in blood serum lipid levels of healthy young men eating controlled mixed diets of common foods, when the linoleic acid content of the dietary fat (a mixture similar to that available in the United States household food supply) was raised from 10 to 20 and 30%.

EXPERIMENTAL PROCEDURE

The study consisted of a 5-day foreperiod followed by a 51-day feeding trial. During the foreperiod, the subjects, maintaining their customary eating habits, recorded their total food intake. During the feeding trial, consisting of 10 5-day periods, controlled diets were prepared and served in the laboratory.

The controlled diets, which were mixed diets similar to that developed by Meyer et al. ('55), adequately supplied all known essential nutrients. They provided daily approximately 13 gm of nitrogen and 3000 Cal. The greater caloric needs of some subjects were met by providing hard sauce patties made of sugar and fat. In the diets and in the hard sauce, fat supplied 40% of the calories. Blends of fat were prepared according to a formula based on the 1955 Household Food Consumption Survey (USDA, '57). The basic blend of fat contained 10% of linoleic acid (analyzed as dienes) and was fed for the first 20 days of the feeding trial. Two other fat blends were made by enriching the basic blend with safflower seed oil. One, containing 20% of linoleic acid, was fed for 15 days. The other, containing 30% of linoleic acid, was fed for the last 16 days of the feeding trial. All subjects received the same proportion of linoleic acid at any one time, and all moved to the next higher level of linoleic acid at the same time.

The subjects were 6 male student volunteers who were examined and declared to be healthy by a physician. They ranged from 20 to 27 years in age, 67.0 to 72.2 inches in height, 62.1 to 83.0 kg in weight. During the feeding trial, quantitative collections of feces and urine were made for each subject.

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ANALYTICAL METHODS

For analytical purposes 5-day composites of food for the group of subjects and 5-day pools of feces and of urine for each subject were prepared. Samples of food and feces were analyzed for fat (AOAC, '55). Samples of food, feces, and urine were analyzed for total nitrogen (AOAC, '50), phosphorus (Fiske and Subbarow, '25), calcium (Ingols and Murray, '49), and magnesium (Orange and Rhein, '51). Daily creatinine excretion in the urine was determined by the method of Clark and Thompson ('49).

Blood was drawn after a fast of 12 to 13 hours on the first and last days of the feeding trial and between changes in the level of linoleic acid in the dietary fat. The frozen serum was shipped to the University of Texas School of Medicine for fatty acid and other lipid analyses. Total lipids from each serum were extracted with a hot 3:1 mixture of ethyl alcohol and ethyl ether and aliquots were taken for the preparation of the total, phospholipid, cholesterol ester, and glyceride fatty acids (Wiese et al., '57). Di-, tri-, and tetraenoic acids were determined by alkaline isomerization. Total cholesterol was recovered from the unsaponifiable fraction of the total fatty acids.

The nitrogen, fat, and mineral intakes of the subjects during the self-selected diet period and the caloric intakes for the entire study were calculated using food composition tables prepared by Watt and Merrill ('50) and McCance and Widdowson ('36). Fatty acid intakes for the self-selected diets and the fatty acids derived from foods other than the fat blends eaten during the feeding trial were calculated from tables prepared by Goddard and Goodall ('59). Fatty acid content of the fat blends was determined by analysis.[3]

RESULTS AND DISCUSSION

Calorie and nutrient intakes. There was considerable variation among the subjects in the nutrient content of their self-selected diets. Their caloric intakes ranged from 2065 to 3425 Cal. per day. Dietary fat supplied 38 to 48% of the calories and contained 6 to 11% of linoleic acid. Calculated nitrogen intakes ranged from 12 to 34 gm per day. Average daily phosphorus, calcium, and magnesium intakes ranged from 1139 to 2511 mg, 543 to 1400 mg, and 189 to 562 mg, respectively.

In the feeding trial the caloric intakes ranged from 2954 to 3342 Cal. per day. Only two subjects (H and I) gained weight, an average of 2.1 kg. Two subjects (J and L), who carried on fairly heavy work, each lost 1.8 kg during the study. The weight of two subjects (K and M) remained unchanged. Because all subjects ate the same quantity of each food except sugar and fat (varied to meet caloric needs), there was no real variation in the intake of nitrogen and minerals among the subjects on any one 5-day period. Average daily intake per subject varied from 12.6 to 13.7 gm of nitrogen, 1051 to 1146 mg of phosphorus, 735 to 785 mg of calcium and from 320 to 375 mg of magnesium. Mean calorie, fat, and fatty acid intakes during the foreperiod and the feeding trial are shown in table 1. The mean fecal fat excretion at each level of linoleic acid during the feeding trial is shown also. Mean intakes of nitrogen, phosphorus, calcium, and magnesium during the study together with the mean retention of each of the 4 nutrients on each level of linoleic acid intake during the feeding trial are found in table 2.

Fecal fat excretion. Average fecal fat per subject for the entire study ranged from 2.6 to 3.9 gm per day. There were highly significant differences among subjects and among levels of linoleic acid intake. Most of the difference among subjects was due to one subject (J) whose fecal fat excretion (2.4, 2.4, and 2.9 gm per day on increasing levels of linoleic acid intake) was significantly lower than any of the others ($P < 0.01$). Average excretions for increasing levels of linoleic acid intake were 3.3, 3.4, and 3.9 gm per day (table 1). With such narrow ranges in quantity of fecal fat, the statistical differences are of doubtful biological significance.

Nitrogen and mineral metabolism. In the balance studies the first 5 days of the feeding trial were regarded as a period of transition. Retention values for nitro-

[3] Personal communication from U. S. Department of Agriculture, Washington, D. C.

TABLE 1

Mean calorie, fat, and fatty acid intakes during the foreperiod and the feeding trial, and fecal fat excretion during the feeding trial (6 subjects)

Dietary period	Calories[1]	Fat					
		Total Fat	Total saturated fatty acids	Oleic acid	Linoleic acid	Linolenic acid	Total fecal fat excretion
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
Foreperiod (self-selected diets)[1]	2666	125.0	49.9	54.8	9.7	0.7	
Feeding trial (controlled diets)[2]							
Linoleic acid in fat, 10.2%	3039	130.6	53.6	62.0	13.3	0.9	3.3
Linoleic acid in fat, 20.4%	2995	130.0	47.7	52.7	26.5	0.8	3.4
Linoleic acid in fat, 30.4%	3017	132.9	43.5	44.2	40.4	0.8	3.9
Standard error of mean							±0.1

[1] Calculated values.
[2] Values for total fat are analyzed values. Values for fatty acids are combinations of calculated and analyzed values.

TABLE 2

Mean intake during the foreperiod and the feeding trial and mean retention during the feeding trial of nitrogen, phosphorus, calcium and magnesium (6 subjects)

Dietary period	Nitrogen		Phosphorus		Calcium		Magnesium	
	Intake	Retention	Intake	Retention	Intake	Retention	Intake	Retention
	gm	*gm*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Foreperiod (self-selected diets)[1]	20.6		1567		949		319	
Feeding trial (controlled diets)[2]								
Linoleic acid in fat, 10.2%	13.4	5.8	1085	119	749	2	328	−38
Linoleic acid in fat, 20.4%	13.1	3.4	1110	93	759	− 9	348	−53
Linoleic acid in fat, 30.4%	13.1	2.4	1076	36	746	−40	343	−27
Standard error of the mean		±0.2		±27		±26		±12

[1] Calculated values.
[2] Analyzed values.

gen, phosphorus, calcium, and magnesium were calculated for a 15-day period on each level of linoleic acid intake for each subject.

Retention of nitrogen, phosphorus, and calcium apparently followed a similar pattern (table 2). Average intake of these three nutrients was higher with the self-selected diets than with the controlled diets. Average retention of each of these nutrients with the controlled diets was highest for the first period (10% of linoleic acid) and successively lower for the higher linoleic acid levels.

Nitrogen balances were positive throughout the study and statistical analyses showed that the subjects did not differ significantly from each other. The statistical analyses showed, however, that there was a significant ($P < 0.01$) linear rela-

tionship (between the proportion of linoleic acid in the diet and the retention of nitrogen). A significant ($P < 0.05$) deviation from this linear relationship was also noted. It seems likely that this deviation might be a flattening out of the regression line. This would coincide with the subjects' approach to nitrogen equilibrium and might indicate the effect of accomodation to changes in nitrogen intake (from the self-selected to the controlled diets) or of uncontrolled factors as well as the effect of linoleic acid.

Differences among subjects were significant in retention of phosphorus ($P < 0.05$) and of calcium ($P < 0.01$). Subject J was in positive balance for both minerals throughout the study. The other subjects fluctuated between positive and negative balance showing a downward trend in the absolute retention of both minerals. Only when phosphorus retention was expressed in terms of body size was there a significant ($P < 0.05$) linear relationship, indicating that as the level of linoleic acid in the dietary fat rose, the amount of phosphorus retained per unit of body size decreased. No such relationship was observed in the calcium retention data. The trend in the calcium data, although parallel to that of phosphorus, was away from equilibrium. It is possible that the feeding of linoleic acid over a wider range of levels might help to elucidate this metabolic trend.

The magnesium pattern of metabolism differed from that of the other three nutrients. Among the dissimilarities was the increase in the average intake from 319 mg per day with the self-selected diets to approximately 340 mg per day with the controlled diets (table 2). There were significant differences ($P < 0.01$) among the subjects in their retention of magnesium. All but subject J were in negative balance most of the time. Studies by Sherman ('52) and Tibbetts and Aub ('37) have shown that a magnesium intake of 300 to 340 mg per day appears to be adequate for normal adult human subjects. Negative balances in these men on intakes of around 340 mg might indicate that the nature of the diets imposed a stress on magnesium metabolism. At present the dearth of information on magnesium requirement and metabolism in man is such that no definite conclusions are justified.

In the magnesium retention data no significant differences were noted among linoleic acid levels. Rather than the consistent downward trend shown by the other nutrients studied, the magnesium data showed a quadratic trend with some evidence of progressing up toward equilibrium. Here again linoleic acid intake over a wider range or for longer periods might have provided some clarification.

The sum of squares of the retention values for each of the 4 nutrients was corrected for the influence of the other nutrients using appropriate partial correlation coefficients (Patterson, '39). Analyses of variance based on the corrected sums of squares did not differ greatly from the corresponding analyses based on the uncorrected data. This indicates that the interaction among nutrients did not mask significantly the effect of increasing the proportion of dietary linoleic acid on the retention of any one nutrient. The only nutrient on which the change in proportion of linoleic acid appeared to have any significant effect was nitrogen. As pointed out earlier, this relationship may have been confounded by other factors affecting the tendency of the subjects toward achieving nitrogen equilibrium following self-selected diets higher in nitrogen content.

Blood serum lipids. In figures 1 and 2 are presented summary charts showing the mean values for lipids in blood serum for the 6 students at the end of the foreperiod (self-selected diets) and at the end of each level of linoleic acid during the feeding trial (controlled diets).

Figure 1 depicts the amount of cholesterol and fatty acids in the total, phospholipid, cholesterol ester, and glyceride fractions. Mean values for cholesterol and for the total fatty acids showed slight increases when linoleic acid constituted 10% of the fat compared to the levels at the end of the self-selected dietary period. Examination of the data for individual students shows this to be the case for all except subject M. Calculated intakes for fat and linoleic acid for subject M on his self-selected diet supplied 40 and 3% of the total calories, respectively. These

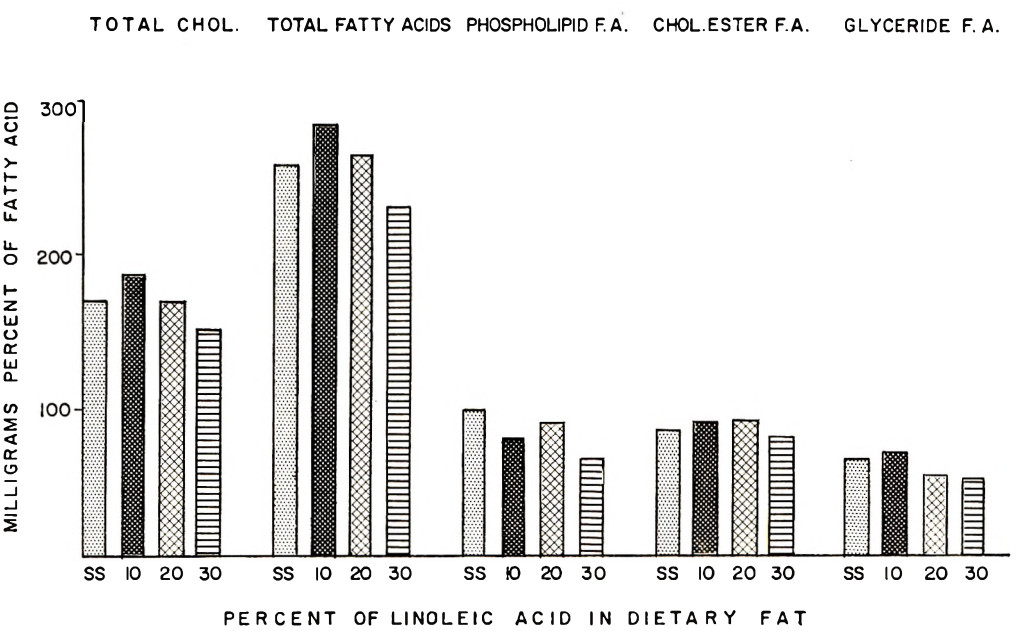

Fig. 1 Mean values for total cholesterol and fatty acid levels in serum lipid fractions for 6 young men receiving varying intakes of linoleic acid; column SS, at end of self-selected dietary regimen, calculated linoleic acid, 6 to 11% in fat.

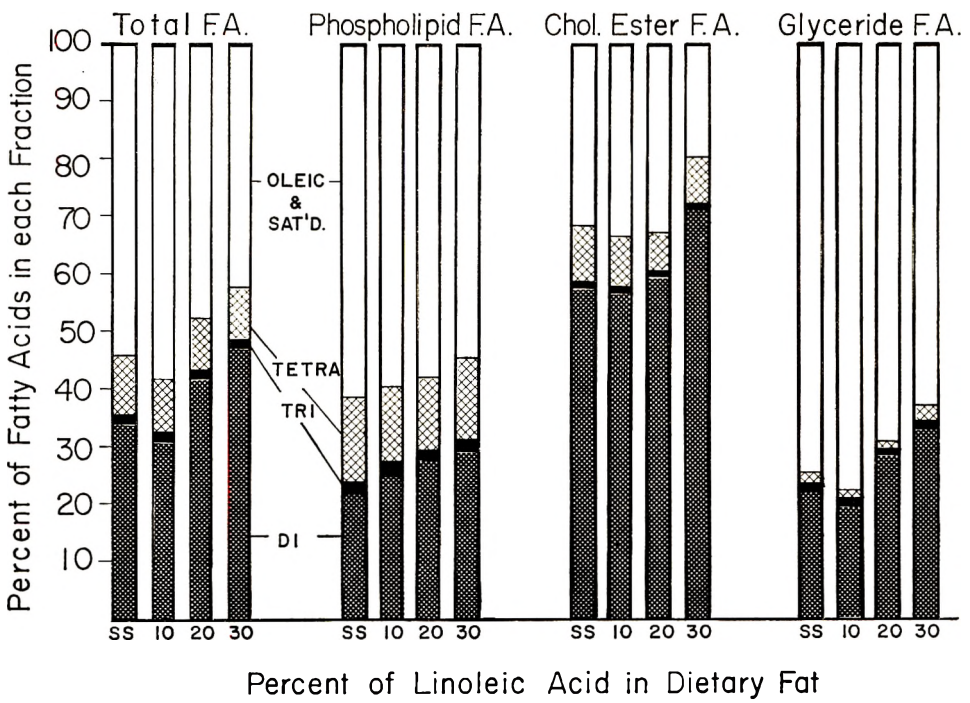

Fig. 2 Distribution of unsaturated fatty acids in total, phospholipid, cholesterol ester and glyceride fractions of serum for 6 young men receiving varying intakes of linoleic acid; column SS, at end of self-selected dietary regimen, calculated linoleic acid, 6 to 11% in fat.

TABLE 3

Summary and comparison of serum lipids for 6 Arkansas students receiving varying amounts of dietary linoleic acid

Diet	Total cholesterol	Total fatty acids				Phospholipid fatty acids				Cholesterol ester fatty acids				Glyceride fatty acids			
			Di-[1]	Tri-[1]	Tetra-[1]		Di-	Tri-	Tetra-		Di-	Tri-	Tetra-		Di-	Tri-	Tetra-
	mg/100 ml	*mg/100 ml*				*mg/100 ml*				*mg/100 ml*				*mg/100 ml*			
Self-selected																	
Mean	170	257	34.2	1.1	10.6	97	21.9	1.5	14.9	84	57.9	0.7	9.9	65	22.8	1.4	2.0
Standard deviation	12.5	29.9	1.5	0.5	1.5	13.3	1.7	0.4	1.5	10.0	1.7	0.2	2.2	12.1	1.3	0.1	0.6
Standard error of mean	5.6	13.3	0.7	0.2	0.7	5.9	0.7	0.2	0.7	4.5	0.8	0.1	0.9	5.4	0.6	0.1	0.3
10% Linoleic in fat																	
Mean	185	285	31.0	1.7	8.9	79	24.9	2.3	13.1	88	57.1	0.9	8.4	71	19.5	1.3	1.4
Standard deviation	22.7	58.9	3.7	0.4	0.8	30.0	1.7	0.6	0.9	8.8	1.3	0.3	1.0	30.3	2.1	0.6	0.5
Standard error of mean	10.2	29.5	1.6	0.2	0.4	13.4	0.8	0.2	0.4	3.9	0.6	0.1	0.4	13.5	0.9	0.3	0.2
20% Linoleic in fat																	
Mean	169	267	42.2	1.0	9.0	89	27.6	1.6	12.3	90	59.8	0.6	6.9	55	28.9	0.8	1.3
Standard deviation	24.9	56.8	2.2	0.4	0.7	11.0	2.1	0.5	0.5	17.3	6.6	0.2	0.5	22.2	3.3	0.3	0.4
Standard error of mean	14.4	32.8	1.3	0.2	0.4	4.9	1.1	0.2	0.2	7.7	2.9	0.1	0.2	9.9	1.5	0.1	0.2

30% Linoleic in fat																
Mean	151	231	47.3	1.3	9.3	64	29.3	1.9	13.7	78	71.3	0.9	8.2	53	36.8	1.0	2.7
Standard deviation	20.2	36.2	2.4	0.1	1.0	28.8	1.9	0.4	2.0	10.2	1.3	0.2	1.4	15.8	1.7	0.2	0.7
Standard error of mean	9.0	16.2	1.0	0.1	0.5	14.4	0.8	0.2	0.9	4.5	0.6	0.1	0.6	7.0	0.8	0.1	0.3
SS vs. 10% Linoleic P[2]	>0.10	>0.10	>0.10	>0.02	>0.05	>0.10	>0.02	>0.02	>0.02	>0.50	>0.10	>0.10	>0.10	>0.50	>0.01	>0.50	>0.10
SS vs. 20% Linoleic P	>0.50	>0.50	<0.001	>0.50	>0.10	>0.10	<0.01	>0.50	>0.01	>0.50	>0.50	>0.50	>0.01	>0.10	<0.01	<0.01	>0.05
SS vs. 30% Linoleic P	>0.05	>0.10	<0.001	>0.10	>0.10	>0.05	<0.001	>0.10	>0.10	>0.10	<0.001	>0.10	>0.10	>0.10	<0.001	>0.01	>0.10
10% vs. 20% Linoleic P	>0.10	>0.50	<0.01	>0.02	>0.50	>0.10	>0.05	>0.05	>0.10	>0.50	>0.10	>0.10	>0.01	>0.10	<0.001	>0.10	>0.50
10% vs. 30% Linoleic P	>0.02	>0.10	<0.001	>0.02	>0.50	>0.10	<0.01	>0.10	>0.50	>0.10	<0.001	>0.50	>0.50	>0.10	<0.001	>0.10	<0.01
20% vs. 30% Linoleic P	>0.10	>0.10	>0.01	>0.10	>0.50	>0.10	>0.10	>0.10	>0.10	>0.10	<0.01	>0.02	>0.05	>0.50	<0.001	>0.10	<0.01

[1] Di- tri- and tetraenoic acids as percentage of fatty acids in each fraction.
[2] P values of < 0.01 are considered significant.

amounts were in the range for the other 5 students which was 38 to 48% and 2 to 4% of the total calories supplied respectively by fat and linoleic acid.

During the feeding trial with progressive increases in the linoleic acid content of the fat there was a definite trend toward a decrease in serum levels for total cholesterol and total fatty acids. A similar trend was observed in the glyceride fraction but not in the phospholipid or cholesterol ester fractions. Quantitative recovery of phospholipids was difficult and may account in part for failure to note a consistent trend in the amount of fatty acid in this fraction.

None of the serum lipid values was considered to be high for healthy adults. During the entire study, cholesterol varied from 117 to 216 mg per 100 ml and total fatty acids varied from 178 to 338 mg per 100 ml. The highest values in both components were observed when linoleic acid constituted 10% of the fat and the lowest when fat contained 30% of linoleic acid which represented caloric intakes of 4 and 12% from linoleic acid.

In figure 2 is shown the distribution of the di-, tri-, and tetraenoic acids in the total, phospholipid, cholesterol ester, and glyceride fractions. The most marked effect of increasing amounts of dietary linoleic acid was reflected in progressive increases in the dienoic acid level in all fractions of the serum. There was little or no effect on the tri- and tetraenoic acid levels which could be related to the dietary intake of linoleic acid. This observation confirms those made on infants and children in which it was found that in the well nourished young human subject the tetraenoic acid level in serum remains relatively constant even though the dietary intake of linoleic acid is increased from 3 to 8% of the total calories (Wiese et al., '54, '58).

The distribution of the di-, tri-, and tetraenoic acids in the fractions of serum was characteristic of the results reported in other studies with healthy human subjects (Okey et al., '60)[4] wherein the highest proportion of dienoic acid in serum occurred in the cholesterol ester fraction and the highest percentage of tetraenoic acid in serum was noted in the phospholipid fraction with little or no tri- and tetraenoic acid in the glycerides.

Although the number of subjects and number of lipid determinations were meager for statistical evaluation, data were subjected to analysis to ascertain whether any of the trends observed were of significance. In table 3 are given the means for all analyses. Even though the levels of cholesterol, total and glyceride fatty acids decreased as the amounts of dietary linoleic acid increased, there were no significant differences for each of these blood serum components between successive increments of dietary linoleic acid. The increasing serum levels in dienoic acid with the increasing linoleic acid intakes, however, were significant in many instances in all fractions of serum at successive increments of linoleic acid in dietary fat. When more data for adult subjects under similarly controlled dietary conditions are examined, a better evaluation of the results reported here will be possible.

SUMMARY AND CONCLUSION

In a study with 6 young men students as subjects, the linoleic acid content was raised from 10 to 20 and 30% of the dietary fat in a mixed diet in which fat provided 40% of the calories. No significant effects of linoleic acid level were observed on the retention of calcium, magnesium, and phosphorus. Magnesium retention showed a quadratic trend as the proportion of linoleic acid in the dietary fat increased. The effect on nitrogen metabolism and fecal fat excretion was not clear cut. There was a downward trend in serum cholesterol and total fatty acid levels with increasing amounts of dietary linoleic acid, but the differences in these serum levels between the increments of linoleic acid intake were not significant. The proportion of dienoic acid in the serum fatty acids increased significantly in all fractions studied as the linoleic acid content of the dietary fat increased.

[4] Wiese, H. F., A. E. Hansen, M. E. Haggard, A. N. Boelsche and H. Davis 1958 Influence of dietary linoleic acid on distribution of unsaturated fatty acids in serum of infants. Federation Proc., 17: 497 (abstract).

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Availability of Amino Acids to Micro-Organisms

III. DEVELOPMENT OF A METHOD FOR COMPARISON OF HYDROLYSATES OF FOODS WITH SYNTHETIC SIMULATED MIXTURES

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The response of single cells to changes in the basal medium offers an economical and speedy method of studying various phases of experimental nutrition that contrasts sharply with the costly and longer experiments with animals and humans. If care is used in interpreting these experiments or the results are tested on animals, clues to the answer of many complex problems in nutrition may be found. Several years ago the authors used a type of microbiological method in a study of the effect of heat and processing on protein in cottonseed meals (Horn et al., '52, '54) which was paralleled by a study of the biological value to rats. This paper deals with an attempt to adapt the method to investigate some interrelationships among amino acids, and also between amino acids and the other nutrients occurring in foods.

To determine the possible presence in the food of unknown growth accelerating or inhibiting factors, a comparison was made of the growth in a standard basal medium containing an acid hydrolysate of the food with the growth when a mixture of synthetic amino acids simulating the protein hydrolysate of the food was added to the same basal medium. Such comparison would verify the analyzed values of the amino acids of the foods, and also indicate the presence of new amino acids or other unknown growth factors in the hydrolysates of the foods.

MATERIALS AND METHODS

In our previous work with cottonseed (Horn et al., '54) we used *Leuconostoc mesenteroides* P–60 as the micro-organism and a medium containing 127 mg of total protein nitrogen per 500 ml in development of a rapid microbial method of determining protein value. Since a hydrolysate of a protein of high biological value containing 127 mg of total nitrogen gave a satisfactory growth curve with this micro-organism, the same basal medium, total nitrogen and organism were adopted for this work.

Solutions prepared by weighing appropriate amounts of the purified commercial amino acids as given in tables 1 and 2 and made up to 500 ml will hereafter be called "synthetic mixtures." If an amount of a protein food equivalent to 127 mg of total nitrogen, such as oats or egg, is hydrolyzed 24 hours, filtered at pH 4 (Horn et al., '53), brought to pH 6.8 and made up to 500 ml, the solution will be called the "food hydrolysate." Portions (1, 2, 3, 4, 5 ml) of the food hydrolysates and of the synthetic mixtures were added to separate tubes together with 5 ml of the standard basal medium (table 3), giving a series of tubes that contained increasing amounts of total nitrogen ranging from 0.254 to 1.270 mg. The tubes were made up to 10 ml with water, autoclaved, cooled and inoculated in the usual way, incubated for 72 hours at 35°C and then titrated (Horn et al., '54). Titration values in terms of milliliters of 0.05 N sodium hydroxide for the 5 levels of nitrogen gave a direct comparison of the growth response to the synthetic mixture and to the food hydrolysate. This is illustrated by results obtained on a hydrolysate of oats and a simulated mixture (table 4, lines 1 to 2).

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TABLE 1

Amino acid pattern for oats (milligrams nitrogen per 500 ml)

Amino acid	N in 127 mg total N	Amino acid	N in 127 mg total N
	mg		*mg*
Isoleucine	3.663	Arginine	16.678
Leucine	6.322	Histidine	4.591
Lysine	5.952	Alanine	5.785
Methionine	1.202	Aspartic	6.680
Phenylalanine	3.841	Glutamic	19.678
Threonine	3.363	Glycine	7.109
Tryptophan	1.413	Proline	5.209
Valine	5.693	Serine	5.918
Tyrosine	2.334		
Cystine	2.787		
	36.570		71.648

	mg	*% total nitrogen*
Total essential amino acid nitrogen	36.570	28.8
Total nonessential amino acid nitrogen	71.648	56.4
Total amino acid nitrogen	108.218	85.2
Ammonium chloride N	18.782	14.8
	127.000	100

TABLE 2

Amino acid pattern for whole egg (milligrams nitrogen for 500 ml)

Amino acid	N in 127 mg total N	Amino acid	N in 127 mg total N
	mg		*mg*
Isoleucine	5.372	Arginine	17.024
Leucine	7.796	Histidine	5.173
Lysine	11.796	Alanine	7.373
Methionine	2.488	Aspartic	8.605
Phenylalanine	3.638	Glutamic	9.834
Threonine	4.904	Glycine	5.337
Tryptophan	1.866	Proline	4.844
Valine	6.291	Serine	8.984
Tyrosine	1.600		
Cystine	2.682		
	48.433		67.174

	mg	*% total nitrogen*
Total essential amino acid nitrogen	48.433	38.1
Total nonessential amino acid nitrogen	67.174	52.9
Total amino acid nitrogen	115.607	91.0
Ammonium chloride N	11.393	9.0
	127.000	100.0

In comparing food hydrolysates with simulated mixtures of amino acids when each was added to the standard basal medium (table 3), 4 factors were found that influenced the growth of the micro-organism. These were: (1) the presence of D-isomers; (2) the presence of ammonium ions; (3) the amount of hydrolyzed starch

TABLE 3

Standard basal medium for comparison of growth curves

Nutrient	Quantity
	gm
Glucose	20[1]
Sodium acetate (anhydrous)	12[1]
Salts A	
K_2HPO_4	1
KH_2PO_4	1
	mg
Salts B	
$MgSO_4 \cdot 7H_2O$	400
$MnSO_4 \cdot 4H_2O$	20
NaCl	20
$FeSO_4 \cdot 7H_2O$	20
Adenine	100
Guanine	100
Uracil	100
Thiamine chloride	2.0
Pyridoxine	0.4
Calcium pantothenate	0.4
Riboflavin	0.4
Nicotinic acid	0.8
p-Aminobenzoic acid	0.4
Biotin	0.01
Folic acid	0.002
Solution brought to 1,000-ml volume, pH 6.8.	

[1] Glucose 1% later raised to 40 gm or 2%; and, concomitantly, the buffer (sodium acetate) increased to 24 gm.

from the food; and (4) the amount of amino acid nitrogen per gram of total nitrogen.

DL-*versus* L-*isomers.* In comparing food hydrolysates and their synthetic counterparts it was soon apparent that the use of DL-amino acids in synthetic mixtures affected the growth of the micro-organisms, especially at high nitrogen levels (table 4, lines 2 and 3). When the vitamins were increased to 5 times the original concentration, the growth noted for the DL-synthetic mixture was much improved at the higher levels of nitrogen (lines 2 and 4). A study of each vitamin separately showed that the greatest stimulating effect on the DL-mixture was caused by extra pyridoxine (lines 2 and 5), whereas extra pyridoxine had no effect on the L-synthetic mixture (lines 3 and 6). Further investigation of the D-isomers revealed that the D-isomers of leucine, isoleucine and valine were causing most of the depression. When the L-compounds of these amino acids were substituted for their DL-isomers, a response similar to that observed for the L-synthetic mixture was obtained without extra pyridoxine (lines 2, 3 and 7). Thus, leaving out the D-isomers of leucine, isoleucine and valine had the same effect as adding extra vitamins (lines 4 and 7). Since the remaining DL-amino acids would still have some effect on the growth at the highest level, L-amino acids hereafter were used exclusively in this work (lines 6 and 7).

Essentiality of the ammonium ion. In determining the essentiality of the ammonium ion, it was found that when all

TABLE 4

Effect on growth response (titration values) of L- *and* DL- *amino acids as synthetic mixtures and comparison of results with food hydrolysates (1% of glucose in basal medium)*

	Hydrolysate	Level				
		1 0.254 mg N	2 0.508 mg N	3 0.762 mg N	4 1.016 mg N	5 1.270 mg N
		ml	*ml*	*ml*	*ml*	*ml*
1	Natural oats	3.4	7.5	11.9	14.7	16.9
2	DL-Synthetic oats	3.9	8.8	12.0	12.9	13.8
3	L-Synthetic oats	4.5	10.0	13.8	15.4	16.9
4	DL-Synthetic oats + 5× all vitamins	—	—	13.5	15.5	16.2
5	DL-Synthetic oats + 5× pyridoxine	4.4	9.3	13.0	14.7	16.1
6	L-Synthetic oats + 5× pyridoxine	4.3	9.5	13.6	15.5	17.2
7	All DL- except leucine, isoleucine, valine	4.3	9.5	13.5	15.5	16.2

the ammonium chloride nitrogen in the media was replaced by nitrogen from all 18 amino acids in the food pattern, the growth was not as good as when ammonium chloride was present. The results of this study are shown in table 5. Maximum growth was not obtained when the ammonium chloride was left out without replacement by other nitrogen (lines 1 and 2). Little improvement was gained when the ammonium chloride nitrogen was replaced by an equivalent amount of a mixture of the 18 amino acids (lines 2 and 3). Diammonium citrate could replace the NH_4Cl, but aspartic acid, glutamic acid, glycine or alanine could not (lines 4 to 8). The minimum amount of ammonium chloride nitrogen was about 10.5 mg (lines 10 to 14). This showed that there are limits in the use of ammonia nitrogen.

Effect of hydrolyzed starch in an oats hydrolysate on the titration levels. In the comparison of whole egg hydrolysate with its synthetic counterpart and with oats hydrolysate it became evident that the amount of hydrolyzed starch in the oats hydrolysate tended to stimulate the growth at higher levels so that it was difficult to compare the hydrolysate with the synthetic mixture which did not contain this extra carbohydrate. Experiments were designed to find the minimum amount of sugar necessary in the basal medium so that addition of hydrolyzed rice starch would have no effect. It was calculated that about 50 mg of hydrolyzed starch was contained in the 5th tube of 5 ml of the food hydrolysate. The effect of increasing the glucose on the titrations of each level when hydrolyzed starch was present or absent is shown in table 6. When 200 mg of glucose were contained in each tube the addition of 4 times the maximum amount of hydrolyzed starch had no effect (lines 1 to 9). Lower titrations were obtained when the glucose was raised to 300 mg per tube (line 10), but the addition of an equal amount of hydrolyzed starch had no extra depressing effect (line 11). Lines 12 and 13 show the titrations obtained with oats hydrolysate when the glucose was raised from 100 to 200 mg per tube. The amount of glucose in the basal medium was therefore raised from 20 to 40 gm per liter and the buffer increased from 12 to 24 gm to take care of extra acid production (table 3).

Amino acid nitrogen. For a study of the amino acid patterns in food, the first simulated mixtures to be made up were those of oats and egg. In calculating the milligrams of each amino acid to give 127 mg of *total* nitrogen, the 18 amino acids were divided into two groups, the first group consisting of 10 amino acids (the 8 amino acids considered essential for adult humans plus cystine and tyrosine) and the second group containing 8

TABLE 5

Comparison of growth on media with ammonium salts and media in which ammonium nitrogen is replaced by amino acid nitrogen

	Hydrolysate	Level				
		1 0.254 mg N	2 0.508 mg N	3 0.762 mg N	4 1.016 mg N	5 1.270 mg N
		ml	*ml*	*ml*	*ml*	*ml*
1	LSO[1] plus 18.8 mg N as NH_4Cl (regular)	4.2	8.9	13.3	15.5	16.9
2	LSO[1] no NH_4Cl	3.5	5.7	8.0	10.2	11.7
3	LSO[1] plus 18 amino acids, oats pattern	4.0	6.8	9.5	11.6	13.0
4	LSO[1] plus diammonium citrate	3.8	9.3	13.7	16.4	17.1
5	LSO[1] plus glutamic acid	3.3	5.3	7.6	9.2	11.0
6	LSO[1] plus aspartic acid	3.4	6.1	8.5	10.4	11.7
7	LSO[1] plus glycine	2.9	5.1	7.2	8.8	10.3
8	LSO[1] plus alanine	3.5	5.9	8.0	10.0	11.5
9	LSO[1] plus 2.62 mg N as NH_4Cl	3.7	7.3	10.2	12.6	16.0
10	LSO[1] plus 5.24 mg N as NH_4Cl	4.0	8.4	11.6	13.7	16.8
11	LSO[1] plus 7.85 mg N as NH_4Cl	4.0	8.6	12.0	14.4	16.8
12	LSO[1] plus 10.50 mg N as NH_4Cl	4.0	8.8	13.1	15.8	16.9
13	LSO[1] plus 13.10 mg N as NH_4Cl	4.2	8.9	13.3	15.7	16.8
14	LSO[1] plus 29.40 mg N as NH_4Cl	4.2	9.1	13.2	15.7	16.6

[1] LSO—Synthetic oats, L-isomers—no ammonium chloride.

amino acids which are considered nonessential for adult humans (tables 1 and 2).

Referring to the calculations accompanying table 1, the total essential amino acid nitrogen of oats was only about 36 mg, or 29% of the total nitrogen; the nonessential amino acid nitrogen was almost 72 mg, or 56% of the total nitrogen. Thus, the amino acid nitrogen was only 85% of the total nitrogen of the oats hydrolysate. The larger part of the remaining 15% of the nitrogen must be ammonia nitrogen coming mostly from amides in the protein and a smaller part from other nonprotein nitrogen of the oats. The character of the nonprotein nitrogen is generally unknown but the amides of glutamic and aspartic acids are the source of most of the extra nitrogen in the hydrolysate which would be present as ammonium chloride. Therefore, ammonium chloride was added to bring the total to 127 mg of total nitrogen.

Contrast these figures with those for egg (table 2). Here the essential amino acid nitrogen was 48.4 mg (or 38%) and the total amino acid nitrogen was 91% of the total nitrogen. This suggests that nutritionally, the superiority of egg to oats might not be due entirely to the pattern of amino acids but to the quantity of essential amino acid nitrogen per gram of total nitrogen.

The use of synthetic simulated mixtures of this type gave an opportunity to compare the pattern of amino acids without interference from extraneous factors which might be present when hydrolysates of foods are compared. The data from growth curves obtained from oats and egg synthetic hydrolysates when made up according to patterns in tables 1 and 2 are shown in table 7 (lines 1 and 2). These

TABLE 6

Effect of hydrolyzed rice starch on titration values

	Hydrolysate	Contents/tube		Level				
		Glucose	Starch	1 0.254 mg N	2 0.508 mg N	3 0.762 mg N	4 1.016 mg N	5 1.270 mg N
		mg	*mg*					
1	LSO[1]	100	—	4.2	10.0	14.1	15.6	17.0
2	LSO[1]	200	—	4.6	10.1	14.7	17.2	18.7
3	LSO[1]	100	600	4.1	10.0	14.5	16.6	18.2
4	LSO[1]	100	—	4.4	9.9	13.7	15.4	16.8
5	LSO[1]	100	100	4.5	10.2	14.5	16.7	17.8
6	LSO[1]	150	—	4.4	10.1	14.5	16.8	18.1
7	LSO[1]	150	150	4.5	10.2	14.8	17.2	18.3
8	LSO[1]	200	—	4.5	10.1	14.7	17.1	18.4
9	LSO[1]	200	200	4.5	10.1	15.0	17.1	18.2
10	LSO[1]	300	—	4.5	9.2	13.3	14.9	15.3
11	LSO[1]	300	300	4.5	9.2	13.4	14.8	15.3
12	Oats	100	—	3.4	7.8	11.9	15.2	17.2
13	Oats	200	—	3.9	8.4	12.9	16.5	19.6

[1] LSO—Synthetic oats, L-isomers.

TABLE 7

Comparison of growth with oats and egg patterns at different nitrogen levels (2% of glucose in basal medium)

	Hydrolysate	Level				
		1 0.254 mg N	2 0.508 mg N	3 0.762 mg N	4 1.016 mg N	5 1.270 mg N
		ml	*ml*	*ml*	*ml*	*ml*
1	Synthetic oats (36)[1]	4.3	10.0	14.7	17.1	18.7
2	Synthetic egg (48)[2]	3.8	8.9	14.5	18.1	19.6
3	Synthetic oats (48)[2]	5.6	13.0	16.7	18.5	19.9

[1] 36.570 mg of essential amino acid nitrogen and 71.648 mg nonessential amino acid nitrogen.

[2] 48.433 mg of essential amino acid nitrogen and 67.174 mg nonessential amino acid nitrogen.

indicated that oats at 36 mg of essential amino acids gave better growth than egg at 48 mg at the two lowest levels, but less growth at the two highest levels of total nitrogen. When the oats pattern was kept for essential amino acid nitrogen and for nonessential amino acid nitrogen separately, but the total essential amino acid nitrogen was raised to 48.43 mg and the nonessential amino acids were lowered to 67.17 mg as in egg, and with the ammonium chloride nitrogen adjusted so that the total nitrogen (127 mg) was kept constant, the growth curves for oats were improved above egg at all levels (line 3). The oats pattern of amino acids in the essential amino acid nitrogen:nonessential amino acid nitrogen ratios used was definitely superior at the first three nitrogen levels and equal or slightly better at the last two nitrogen levels. Results indicate that total essential amino acid nitrogen per gram of total nitrogen, as well as the amino acid pattern, was a factor in the growth of this micro-organism and comparing nutritive value of proteins on a total nitrogen basis alone may lead to erroneous conclusions.

DISCUSSION

Eagle ('58) points out the extraordinary parallelism in the amino acid and vitamin requirements of animal cells and Lactobacilli and predicts that the pathways by which the nutrients are metabolized to yield the cellular macromolecules will probably prove no less similar. Some workers (Eagle, '59; Gale and Van Halteren, '51) have pointed out that the degree to which any amino acid can be concentrated by the cell varies with the environment, and Gale (Gale and Van Halteren, '51) has shown that various mixtures of amino acids outside the cell determine the amount of any one amino acid that accumulates inside the cell.

It is therefore reasonable to assume that changes in concentration of an amino acid in the media, interactions of the various nutrients, and the presence of any new factors outside the cell would be reflected in the growth of the cells and the utilization of the amino acids inside the cell.

The effect of extra pyridoxine or of pyridoxal phosphate on DL-amino acid can be interpreted from the findings of Holden and Snell ('49) and those of Armstrong et al. ('50). These workers observed that pyridoxine bears some specific relation to the metabolism of D-amino acids in animals as well as to bacteria. In experiments on rats, Wretlind ('56) showed that omission of the D-forms of leucine and isoleucine from diets containing D-valine resulted in growth stimulation. Wachter and Berg ('60), working with the rat, have reported that replacement of a group of L-isomers of essential amino acids (isoleucine, leucine, lysine, threonine and valine) by their DL-forms at twice the L-level produced less rapid growth than a diet of L-essential and nonessential amino acids.

In our experiments using L-amino acids exclusively, ammonia nitrogen apparently could not be obtained from these isomers. There is evidence from studies in progress that some ammonium nitrogen can be supplied by D-isomers when DL-amino acids are used. Evidence for the utilization of ammonia N by growing rats has been reported by Lardy and Feldott ('50) and by Rose et al. ('49). These studies confirm the observations of Foster et al. ('39) with respect to the metabolic availability of labeled ammonia. Frost and Sandy ('51) using a large excess of essential amino acids noted that replacing a part of the nitrogen of the essential amino acids by various sources of nitrogen gave a much greater response than given by the essential amino acids alone. When 20% of the total nitrogen was present as nitrogen other than that of essential amino acids the best utilization occurred. They also pointed out that when DL-essential amino acids are used, it cannot be assumed that the D-isomer can serve as a supply of nonessential amino acid nitrogen. Eagle ('59) working with human cells has found glutamine to be an essential amino acid that cannot be replaced by glutamic acid, but the cells do have a limited capacity to make glutamine from glutamic acid and ammonia nitrogen.

It must be emphasized that we are dealing here with complete hydrolysates and the question of differences in rates

of digestion of the foods is not a factor, as it might well be if the unhydrolyzed protein is fed as is done in animal work.

SUMMARY

A microbiological method has been developed by which food hydrolysates can be compared with synthetic mixtures of amino acids to simulate the protein hydrolysate when each is added to a standard basal medium. In the development of this method it was found that amount of amino acid nitrogen, D-isomers, ammonium ions, and carbohydrate content all had some influence on the growth curves of *Leuconostoc mesenteroides* P–60. When the simulated mixture was adjusted to take care of these factors, a useful and satisfactory mixture could be obtained by which differences from the corresponding food hydrolysate could be detected.

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Effects of Intake and Calcium to Phosphorus Ratio on Absorption of These Elements by Sheep[1]

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Although there is ample evidence with laboratory animals indicating that the Ca:P ratio in the diet is important, the evidence with ruminants does not consistently indicate detrimental effects from ratios deviating widely from that thought to be optimum. It is commonly recommended that Ca:P ratios in the diets of laboratory animals be between 2:1 and 1:2 although with adequate vitamin D, ratios outside these limits may be satisfactory (Maynard and Loosli, '56). With dairy cattle, ratios up to 10:1 have produced no ill effects on milk production and calcium and phosphorus balance (Haag et al., '32; Lamb et al., '34). Using body weight gains of beef cattle as the criterion of response, Dowe et al. ('57) observed that a Ca:P ratio of 9.1:1 resulted in significantly lower gains than a ratio of 4.3:1. The inorganic phosphorus content of the plasma, however, was not depressed below a level considered to be adequate. It was therefore concluded that excess calcium depressed gains by a detrimental effect upon nutrients other than phosphorus. Marston ('39) reviewed the early work and Duncan ('58) has reviewed and summarized more recent work on the role of calcium and phosphorus in ruminant nutrition. There is not complete agreement on the relative effect of amount and of the ratio of calcium to phosphorus upon absorption and excretion of these two elements. Since much of the earlier work on this and related problems was done using gains in body weight, balances and blood levels as measures of response, it seemed desirable to study directly the effect of various ratios of calcium to phosphorus upon absorption using the isotope dilution technique for measuring absorption. This paper reports the results of such studies.

EXPERIMENTAL

Experiment 1. In order to establish detailed techniques for simultaneous use of Ca^{45} and P^{32} in the dilution technique, it was necessary to conduct a preliminary investigation. The technique being used at this station for phosphorus availability studies (Lofgreen and Kleiber, '54) involves one subcutaneous injection of a phosphate solution containing P^{32} seven days prior to the initiation of the collection of blood and feces. A preliminary investigation was designed to study the feasibility of using only one subcutaneous injection of a $Ca^{45}Cl_2$ solution and to determine whether the concentration of P^{32} in the saliva could be used as an indication of the concentration in the blood.

Five mature wethers were fed a maintenance level of pelleted mixed grass-legume hay containing 0.96% of calcium and 0.39% of phosphorus. Each wether was given one subcutaneous injection of a calcium chloride solution containing 3 mc of Ca^{45}. All wethers were then placed in collection stalls and daily samples taken of blood and feces. Ten days after Ca^{45} administration each wether received a subcutaneous injection of a solution of potassium phosphate containing 5 mc of P^{32}. Collection of blood and feces was continued for 21 days. Daily saliva samples were withdrawn from the mouth of each sheep by using a copper tube connected to a 50-ml collection flask and to a water aspirator. This method proved to

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be easy to handle and efficient to use. A 20-ml sample of saliva could be collected in approximately 4 minutes. Such a technique proved to be superior to a sponge gag technique.

Blood samples were allowed to clot and the serum saved for analyses. Serum rather than plasma was used since no difference was observed in the specific activity (microcuries of isotope per milligram of total element) of the calcium and phosphorus in serum and plasma. Calcium and phosphorus were determined quantitatively by the methods of Clark and Collip ('25) and Sumner ('44), respectively. Using essentially the technique of Comar et al. ('51), serum calcium was precipitated as calcium oxalate into steel cup planchets for radiocalcium determination. The supernatant containing the serum phosphorus, the saliva and fecal samples were ashed prior to preparation of planchets so that radioactivity could be concentrated for ease and accuracy in counting. Radiophosphorus in the serum and saliva was determined on the ash solution by the technique described by Kleiber et al. ('51). Planchets of fecal and saliva ash solutions were counted with and without aluminum shields to determine the concentration of each isotope.

Experiment 2. Having established suitable methods, the main trial was designed to study the effects of dietary Ca:P ratio on absorption. Fifteen wether lambs approximately 6 months old were randomly assigned to three groups of 5 lambs each. The three groups were fed rations containing Ca:P ratios of approximately 1:1, 3:1 and 6:1. The element present in the lowest amount was sufficient to meet the requirements established by the National Research Council ('57). The basal ration contained 55% of mixed grass-legume hay, 27% of ground barley, 9% of molasses dried beet pulp and 9% of linseed oil meal. The approximate ratios of 1:1, 3:1 and 6:1 were achieved by adding to the basal ration 1% of monosodium phosphate and 1 and 3.5% of calcium carbonate, respectively. All lambs were fed individually and received their respective rations three weeks prior to the initiation of the balance studies.

One subcutaneous injection of approximately 3 mc of Ca^{45} and one of approximately 5 mc of P^{32} were given each lamb and fecal samples collected daily for three days in order to establish the time lag when the peak activity in the blood is reflected in the feces. This procedure is necessary to ascertain the correct time interval between taking blood and fecal samples (Lofgreen, '60). Ten days following isotope injections, blood and fecal samples were collected for 7 days, and the amount of calcium and phosphorus absorbed determined from the ratio of the specific activity of the feces to that of the serum (Lofgreen and Kleiber, '53, '54). The same chemical and ratio assay procedures were used as in experiment 1.

RESULTS AND DISCUSSION

In experiment 1 it was found that 10 days after one subcutaneous injection of either Ca^{45} or P^{32} a rather constant relationship was reached between the specific activity of the serum and the feces. The decline in activity for the next 10 days could be considered to be linear. This observation demonstrates that a simultaneous injection of Ca^{45} and P^{32} can be made and the collection period initiated 10 days following injection. The maximum concentration of both isotopes in the blood serum occurred within the first hour after injection.

Study of the relationship of the specific activity of the phosphorus in saliva to that in the blood indicated that saliva can be used after a 10-day preliminary period as an indication of the specific activity of serum phosphorus. The mean specific activity of both blood serum and saliva was 6.6 during a 10-day collection period. Variability, however, was somewhat greater in the case of saliva (coefficient of variation = 9.1 for serum and 13.4 for saliva). Specific activity of saliva calcium was not determined due to copper contamination obtained in the collection process. Saliva contained sufficient Ca^{45} for accurate counting, but the low concentration of calcium in the saliva may limit its value for estimating serum calcium activity. Blood serum samples were used for determination of calcium and phosphorus specific activity.

The relationship of intake to absorption and metabolic fecal excretion of calcium and phosphorus in experiment 2 is shown in figure 1. As either calcium or phosphorus intake increased the amount absorbed increased. Metabolic fecal excretion of calcium, however, remained almost constant, whereas each increase in phosphorus absorbed was accompanied by an increased metabolic fecal excretion. If a low urinary excretion of Ca and P is assumed (Lofgreen, '60) it appears that increased calcium absorption caused increased retention whereas with phosphorus, increased absorption resulted in greater excretion with no increase in retention. Whether this indicates a basic difference in the excretion of the metabolic fecal calcium and phosphorus cannot be ascertained from this study. It may be merely that the calcium absorbed was not sufficient to meet the calcium requirement and each increase in intake resulted in an increase in the amount absorbed and retained. Phosphorus absorbed when the 2.8:1 and 6.0:1 ratios were used, however, may have been sufficient to meet the requirements and therefore the increased absorption obtained on the 0.8:1 ratio was excreted. Since the urine was not collected in these studies, however, one cannot be certain that some of the increased calcium absorbed during the higher intakes was not excreted in the urine although it appears unlikely that a significant amount would be excreted in this manner. As evidence that the amount of calcium absorbed may not have been sufficient to meet the requirements of the body, an average of only 11% of the dietary calcium was absorbed, whereas an average of 55% of the phosphorus was absorbed. Although calcium absorption is sometimes affected by the calcium needs of an animal (Visek et al., '52; Hansard et al., '54), for the animals in this study there were no significant differences in the percentage of the calcium intake which was absorbed, perhaps indicating poor availability of the calcium in all rations.

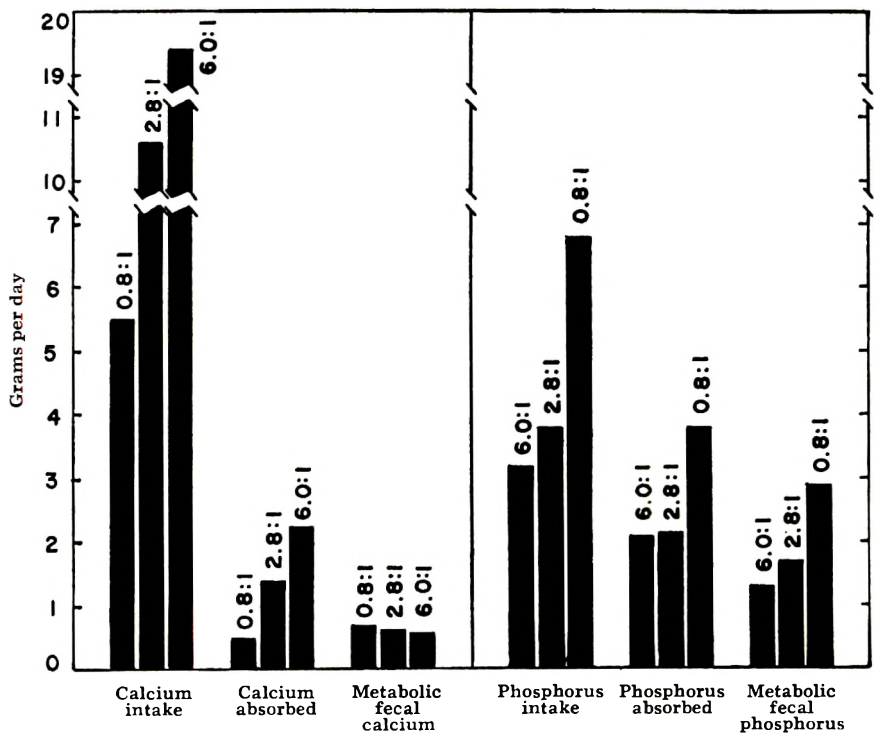

Fig. 1 Mean calcium and phosphorus intake, absorption and metabolic fecal excretion for animals in experiment 2.

Because the intakes of calcium and phosphorus varied as well as the ratios, it is diffcult to tell whether the increases in absorption are due to the effect of ratio or merely the result of increased intake. It is possible, however, to adjust the amount absorbed with the three ratios to an equal intake by an analysis of covariance (Snedecor, '56) when there is a significant relationship between intake and absorption. A significant difference existing among means adjusted to an equal intake would indicate an effect of Ca:P ratio. The results presented in table 1 show a highly significant regression of calcium and phosphorus absorbed during the corresponding intake of these. In both cases there was a highly significant difference among the means before adjusting for differences in intake ($P < 0.01$). When adjusted to an equal intake, however, there were no differences among the means. All the differences in absorption were therefore due to differences in intake and Ca:P ratio was without effect upon absorption of either calcium or phosphorus. The regression of metabolic fecal calcium excretion on calcium absorbed was not significant, indicating no effect of either intake or ratio. A highly significant regression of metabolic fecal phosphorus excretion on phosphorus absorbed was noted and a highly significant difference among the unadjusted means. When adjusted to equal amounts of phosphorus absorbed, there still existed a significant difference in the mean excretion of metabolic fecal phosphorus for the three ratios. This indicates that Ca:P ratio exerted an influence upon the excretion of metabolic fecal phosphorus even though it did not affect absorption. In other words, the amount of calcium absorbed is important in governing the metabolic fecal excretion of phosphorus. Since both calcium and phosphorus exert an influence on metabolic fecal phosphorus excretion a multiple covariance analysis was performed to adjust for both. Table 2 presents the results.

This analysis shows that the amount of calcium absorbed is not influenced by phosphorus intake nor is the amount of phosphorus absorbed influenced by the calcium intake. Metabolic fecal calcium excretion in this study was not influenced by either the calcium or phosphorus absorbed, whereas the excretion of metabolic fecal phosphorus was significantly influenced by the amount of both calcium and phosphorus absorbed. When adjusted to an equal amount of calcium absorbed as well as to equal phosphorus absorbed, there is no longer a significant difference among the mean metabolic fecal phosphorus excretion on the different ratios indicating all the differences are accounted for by a consideration of both calcium and phosphorus absorbed. A comparison of the standard partial regression coefficients furnishes information on the relative effect of the absorbed calcium and phosphorus. The amount of calcium absorbed exerts about half as much influence on metabolic fecal phosphorus excretion as the amount of phosphorus absorbed but the effect is in an opposite direction. In other words, an increase in phosphorus absorbed causes an increase in metabolic fecal phosphorus excretion which tends to be offset when more calcium is absorbed. Thus the smallest metabolic fecal phosphorus excretion occurred when using the 6.0:1 ratio. Table 3 shows that the ratio of the absorbed calcium to absorbed phosphorus was approximately 1:1 when the

TABLE 1

Results of covariance analysis

Regression of interest	Regression coefficient	Significance of F for unadjusted means	Significance of F for adjusted means
Calcium absorbed on calcium intake	0.12*	$P < 0.01$	$P > 0.05$
Phosphorus absorbed on phosphorus intake	0.53*	$P < 0.01$	$P > 0.05$
Metabolic fecal calcium excretion on calcium absorbed	−0.03	$P > 0.05$	
Metabolic fecal phosphorus excretion on phosphorus absorbed	0.72*	$P < 0.01$	$P < 0.05$

* Indicates coefficients are significant at the 5% level.

TABLE 2

Results of multiple covariance analysis

Regression of interest	Standard partial regression coefficients	Significance of F for adjusted means
Calcium absorbed (Y) on calcium intake (X_1) and phosphorus intake (X_2)		
Y on X_1 independent of X_2	0.52*	
Y on X_2 independent of X_1	−0.40	
Phosphorus absorbed (Y) on phosphorus intake (X_1) and calcium intake (X_2)		
Y on X_1 independent of X_2	1.13**	
Y on X_2 independent of X_1	0.36	
Metabolic fecal calcium excretion (Y) on calcium absorbed (X_1) and phosphorus absorbed (X_2)		
Y on X_1 independent of X_2	−0.30	
Y on X_2 independent of X_1	−0.13	
Metabolic fecal phosphorus excretion(Y) on phosphorus absorbed (X_1) and calcium absorbed (X_2)		
Y on X_1 independent of X_2	0.65**	
Y onX_2 independent of X_1	−0.37**	$P > 0.05$

*,** Indicates coefficients are significant at the 5 and 1% levels, respectively.

TABLE 3

Relationship of calcium and phosphorus intake, absorption, metabolic fecal excretion and apparent digestion

Item of interest	Ca:P ratio		
	0.8:1	2.8:1	6.0:1
Intake, gm/day			
Calcium	5.51[1]	10.57[2]	19.35[3]
Phosphorus	6.79[1]	3.82[2]	3.21[2]
Ca:P ratio	0.8:1	2.8:1	6.0:1
Absorption, gm/day			
Calcium	0.48[1]	1.39[2]	2.25[3]
Phosphorus	3.80[1]	2.12[2]	2.09[2]
Ca:P ratio	0.13:1	0.66:1	1.1:1
Metabolic fecal excretion, gm/day			
Calcium	0.71[1]	0.64[1]	0.60[1]
Phosphorus	2.91[1]	1.68[2]	1.32[2]
Ca:P ratio	0.24:1	0.38:1	0.45:1
Apparent digestion, gm/day			
Calcium	−0.23[1]	0.75[2]	1.65[3]
Phosphorus	0.89[1]	0.44[1]	0.77[1]
Ca:P ratio	−0.26:1	1.7:1	2.1:1

[1,2,3] Values having common superscripts are not significantly different according to Duncan's multiple range test (Duncan, '55).

ratio in the diet was 6.0:1. As shown in table 3 the Ca:P ratio absorbed varied from 0.13:1 to 1.1:1 even though the ratio in the diet varied from 0.8:1 to 6.0:1. It is possible that some of the lack of agreement on the importance of Ca:P ratios in the diets of ruminants may be a result of the lack of absorption of either of the elements resulting in a quite different ratio absorbed than that which was fed. Since no effect of Ca:P ratio on absorption was observed in these studies but rather an effect of the absorbed calcium and phosphorus upon phosphorus excretion, the ratio in the diet will have no effect upon utilization unless it is absorbed.

SUMMARY

Feeding rations containing Ca:P ratios of 0.8:1, 2.8:1 and 6.0:1 to growing lambs had no effect on the amount of calcium or phosphorus absorbed. The amount of either calcium or phosphorus absorbed was directly related to the amount fed. Excretion of metabolic fecal calcium was independent of the amount of either calcium or phosphorus absorbed since it remained essentially constant on the three ratios. Metabolic fecal phosphorus excretion, however, was significantly affected by both calcium and phosphorus absorbed. Within the ranges studied the amount of metabolic fecal phosphorus excreted increased as the phosphorus absorbed increased and decreased with increasing calcium absorption. The smallest excretion of metabolic fecal phosphorus occurred with the ration containing a 6.0:1 Ca:P ratio. The ratio of absorbed calcium to phosphorus with this ration, however, was 1:1.

The specific activity of the phosphorus of the saliva was observed to be a good indication of the specific activity of the phosphorus of blood serum 10 days after a subcutaneous injection of P^{32}.

ACKNOWLEDGMENTS

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Avian Disease Virus and Nutrition Relationships

II. EFFECT OF DIETARY ENZYMES AND LYSINE ON THE GROWTH OF WHITE LEGHORN CHICKS INFECTED WITH NEWCASTLE DISEASE VIRUS

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Squibb ('61) has shown that Newcastle disease virus (NDV) restricts feed and water intake and depresses growth of susceptible chicks. Vitamin A therapy during NDV involvement had no effect on the course of the infection. Dietary enzymes have been investigated chiefly as feed supplements and have been found to be beneficial in barley rations for poultry (Arscott and Rose, '60; Willingham et al., '60). The essential nature of dietary lysine in the chick diet is well established. Data are lacking, however, on the value of dietary enzymes and lysine in poultry rations during periods of disease stress.

The studies reported here were undertaken to observe the effects of dietary enzyme and lysine supplementation of a simplified low-lysine basal ration on the growth of non-infected and NDV-infected White Leghorn chicks.

PROCEDURES AND RESULTS

Procedures common to all experiments consisted of housing the infected and non-infected birds in all-wire batteries in separate, isolated, air-conditioned rooms maintained with a diurnal temperature range of 68° to 72°F. Test diets and water were offered ad libitum. The birds were weighed individually zero, three, 7, 10 and 14 days following NDV inoculation. The first three days following inoculation were considered the "incubation period" of the virus; three to 7 days, the period of "active involvement"; and 7 to 14 days, the period of "initiation of recovery." These periods, procedures for virus preparation and inoculation, and confirmation of NDV infection were described previously by Squibb ('61).

The same simplified low-lysine basal ration containing 21.3% of crude protein was used for each trial. This diet, by calculation, contained one-half the lysine requirement of the chick and consisted of sesame oil meal, 40.0; ground yellow corn, 57.1; calcium carbonate, 1.0; ground bone meal, 1.0; sodium chloride, 0.5; minor elements,[1] 0.2; and a vitamin premix,[2] 0.2. For supplementation of the basal ration, L-lysine was added at 0.5% of the diet, an amount calculated adequate to make the diet complete in all essentials.

Statistical analyses and tests of significance of data were adapted from Snedecor ('57).

Experiment 1. The object of this trial was to observe the effect of supplementing a low-lysine basal ration with lysine and a crystalline trypsin on the growth of chicks during NDV infection. Day-old White Leghorn cockerels were maintained with the low-lysine basal diet for 24 days and then allotted to 6 experimental groups of 25 birds each. Treatments were as follows: group 1, basal diet; group 2, basal + lysine; group 3, basal + NDV; group 4, basal + lysine + NDV; group 5, basal + 0.01% trypsin + NDV; and group 6, basal + lysine + 0.01% of trypsin + NDV.

Newcastle disease virus infection had little effect on growth during the period designated "incubation" as shown in figure 1. A significant ($< 1\%$) depression of growth was noted during the "active involvement" period in the lysine and lysine + trypsin supplemented groups. Although a growth depression was evident

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[1] Squibb ('61).

[2] See footnote 1.

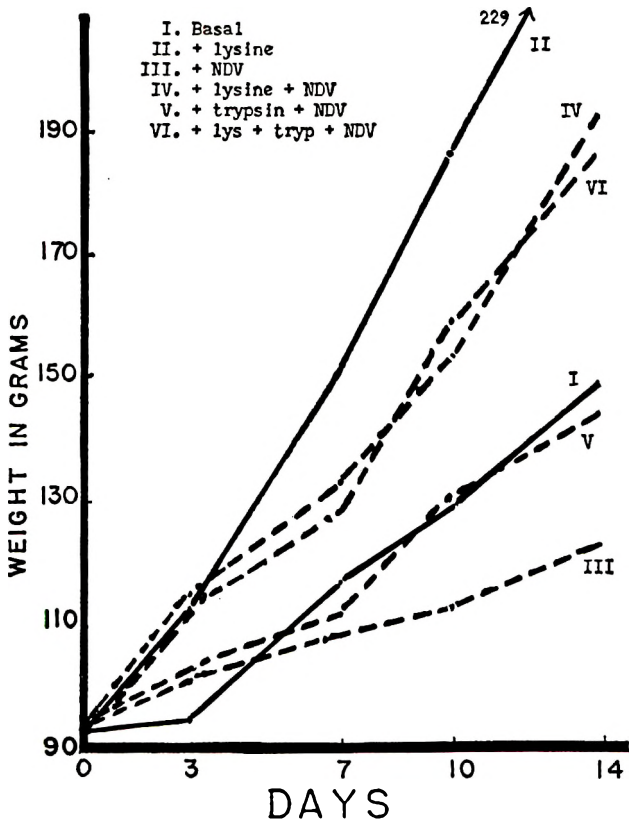

Fig. 1 Effect of lysine and trypsin on the growth of immature White Leghorn cockerels during a 14-day Newcastle disease virus infection period.

in the infected groups fed the basal or trypsin supplemented rations, the effect was non-significant. At the end of the experiment, the growth of the infected birds in the basal and lysine supplemented groups remained significantly ($< 1\%$) depressed. The infected group fed trypsin, however, was similar to the non-infected chicks on the basal ration.

Experiment 2. This trial replicated in part the treatments of experiment 1; in addition, the basal diet was supplemented with a partially purified bacterial amylase free of protease. All chicks were depleted by using the low-lysine basal ration for a 24-day period and were then divided into 8 groups of 20 birds each as follows: group 1, basal diet; group 2, basal + lysine; group 3, basal + NDV; group 4, basal + lysine + NDV; group 5, basal + 0.01% of trypsin + NDV; group 6, basal + 0.1% of amylase + NDV; group 7, a standard chick starter (Squibb, '61); and group 8, chick starter + NDV.

The data of figure 2 show that in this experiment growth depression resulting from NDV infection during the "active involvement" period was similar to the results observed in experiment 1. Furthermore, the growth of the group fed the standard chick starter diet was significantly ($< 1\%$) depressed during this period. At the end of the experiment, NDV infection had again significantly ($< 1\%$) depressed the growth of the basal and supplemented groups as well as of the birds fed the standard chick starter ration. Growth of the NDV-infected birds on the diets to which trypsin and amylase were added was slightly better than that of the infected basal group.

Experiment 3. Based on the results of experiment 2, the effects of lysine and amylase supplementation on the growth

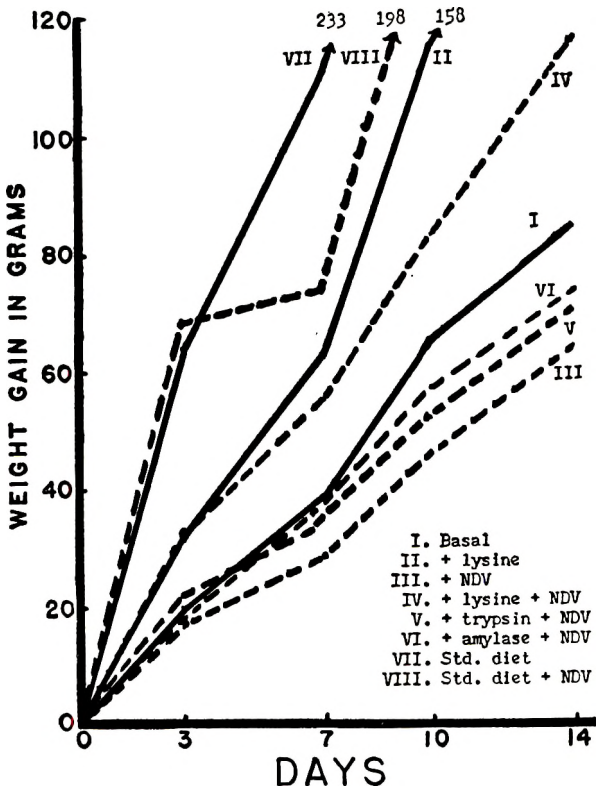

Fig. 2 Effect of lysine, trypsin and amylase on the growth of immature White Leghorn cockerels during a 14-day Newcastle disease virus infection period.

of susceptible chicks infected with NDV were studied further. In this trial, however, the chicks were not depleted with the basal ration; rather, 180 day-old cockerels started to receive immediately the test rations and continued on these feeding regimens up to and following inoculation with the virus. Each group of 45 day-old chicks was fed as follows: group 1, basal diet; group 2, basal + lysine; group 3, basal + 0.1% of amylase; and group 4, basal + amylase + lysine. When the chicks fed these diets reached 28 days of age 10 of each of the 4 groups were retained as non-infected controls and the remaining 35 birds were inoculated with NDV.

In the non-infected chicks, the addition of L-lysine or amylase to the basal ration significantly ($< 1\%$) increased growth in comparison with the group fed the basal ration alone (fig. 3). In the group fed both lysine and amylase, however, the growth response of the chicks was significantly ($< 1\%$) lower than that in the group given lysine alone.

Newcastle disease virus infection again significantly ($< 1\%$) depressed the growth of the birds in all treatment groups during the periods of "active involvement" and "initiation of recovery"; this growth depression was proportionately greater when the low-lysine basal rations were supplemented with L-lysine.

DISCUSSION

These studies, confirming a previous report (Squibb, '61), showed that during the period designated "incubation," which included the first three days following inoculation, NDV infection had little effect on chick growth. Greatest growth depression occurred during the "active involvement" stage, or three to 7 days post inoculation. During "initiation of recovery," 7 to 14 days after inoculation, a normal rate of growth was observed in all birds with the exception of those with acute pa-

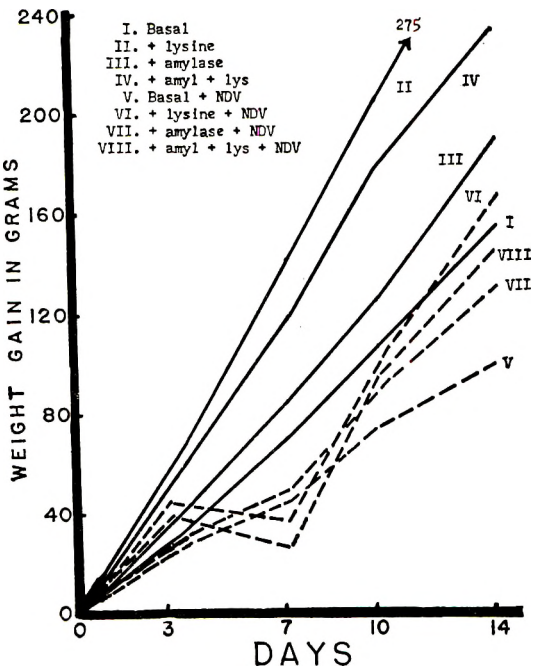

Fig. 3 Comparative growth rates of controls and Newcastle disease virus infected immature White Leghorn cockerels supplemented with lysine and amylase.

ralysis. Although NDV depressed growth in all infected birds, the greatest magnitude of difference was observed in the groups fed rations calculated to be complete in all dietary essentials. This magnitude was evident not only from examination of actual growth rates but also when rate of gain of an infected group was expressed in ratio to the potential gain of its non-infected control. The depressing effect of the disease, however, was reduced when the imbalanced basal ration was fed alone or supplemented with dietary enzymes. This was especially so when the test diets were fed for three- to 4-week periods prior to NDV inoculation.

These data would indicate that NDV attains greatest involvement in the presence of normal cellular metabolism as would be the case in well-fed, growing birds. In contrast with the extent of involvement in well-fed birds, the depletion-repletion technic prior to inoculation provides a less suitable host for the virus. The growth depression observed when the NDV-infected birds were fed the basal ration supplemented with lysine cannot be attributed to this amino acid *per se* since even greater growth depressions resulted when a normal chick diet was provided (fig. 2 and Squibb, '61).

The lower growth rate observed when the basal ration was supplemented with both lysine and amylase cannot be explained by the data here; nor can the increased growth noted in the infected and non-infected birds fed either crystalline trypsin or the protease-free amylase, an effect apparent only when these ingredients were added to the imbalanced low-lysine basal ration.

SUMMARY

Newcastle disease virus (NDV) involvement, in the immature White Leghorn cockerel, resulted in a greater growth depression when complete rations rather than those imbalanced by deficiencies of lysine were supplied.

Trypsin or amylase supplementation of a low-lysine diet resulted in increased growth of NDV-infected chicks. This phenomenon was observed, however, only when the experimental rations were deficient in lysine.

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Some Relationships Between Caloric Restriction and Body Weight in the Rat

I. BODY COMPOSITION, LIVER LIPIDS AND ORGAN WEIGHTS[1]

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It has been shown that when the caloric consumption of young rats is restricted to such a level that body weight remains constant, the amount of food required per day decreases over a period of 4 to 6 weeks and then becomes constant for several weeks. Quimby ('48) observed that the caloric requirement for weight maintenance of 50-gm rats fed a synthetic stock ration decreased to about one half of the original value in 5 weeks. Similar results were obtained by Kaunitz et al. ('56), who measured the caloric requirements for weight maintenance of young rats fed diets, high in protein, carbohydrate, or fat. Although the rate and the degree of decrease of caloric requirements depended on the particular diet fed, the general pattern was the same: a progressive decline during the first 5 weeks.

This caloric restriction represents a stress to which the animals have become adapted through one or more metabolic alterations. The mechanisms responsible for this adjustment have not been extensively investigated. It has been suggested that there is an increase in intestinal absorption, but it is difficult to envision an increase which would be sufficiently large to account for these observations. It has also been suggested that there is a decreased basal metabolic rate. Keys and co-workers ('50) have shown that the basal metabolic rate declines during complete starvation. McCance and Mount ('60), however, found no significant change in oxygen consumption of pigs subjected to caloric restriction without weight loss.

The experiments described here were designed in order to investigate the relationship between caloric requirements and weight maintenance and some of the possible mechanisms by which this adaptation occurs. The re-establishment of caloric requirements following a period of severe food restriction associated with weight loss was also studied. The composition of the body and the content of lipid in the liver were examined as possible factors in the adjustment of caloric requirements (caloric-requirement-adaptation).

METHODS

Animals and diet. The experiments were carried out on male rats of the Long-Evans strain, obtained from an established commercial dealer. When received the animals weighed 150 to 200 gm. They were housed in individual cages with wire bottoms and fed a purified diet, the composition of which is shown in table 1. The vitamin supplements were fed separately from the remainder of the diet and the amounts were independent of the quantity of diet consumed. When the animals reached a weight of approximately 200 gm, the dietary restrictions were initiated.

The animals were grouped in the following manner:

Experiment 1

Body composition

Group 1 (zero-time controls)—these animals were killed when they had attained

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TABLE 1

Composition of synthetic diet[1,2]

	%
Vitamin-free casein[3]	18
Cottonseed oil	8
Powdered sucrose	66.9
Cellulose	3
Salt mixture[4]	4
Choline	0.1

[1] In addition to the above diet each animal received a supplement which consisted of the following vitamins: (in mg/week) thiamin, 0.3; riboflavin, 0.3; niacin, 1.2; pyridoxine, 0.4; Ca pantothenate, 1.2; menadione, 0.4; biotin, 0.06; folic acid, 0.06; vitamin B_{12}, 0.0015; vitamin A, 500 I.U.; irradiated ergosterol, 500 I.U.; and α-tocopherol, 2.5 mg. The vitamin supplements were fed separately from the remainder of the diet and the amounts were independent of the quantity of diet consumed.

[2] The experimental diet yielded about 4.14 Cal./gm, using calorigenic values of 4, 4, and 9.2 for carbohydrate, protein, and fat, respectively.

[3] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.

[4] For the composition, see mixture no. 3 (Hawk et al., '54).

a weight of 200 gm; they were not subjected to caloric restriction.

Group 2 (R)—these animals were subjected to caloric restriction so that they remained at 200 gm; killed after 6 weeks.

Group 3 (RR)—held at 200 gm for 14 weeks.

Group 4 (RRR)—held at 200 gm for 26 weeks.

Group 5 (RRA)—held at 200 gm for 14 weeks and then fed ad libitum for 6 weeks.

Group 6 (RS)—held at 200 gm for 6 weeks, then brought down to 130 gm by more severe caloric restriction; killed after 5 weeks at 130 gm.

Group 7 (RSR)—held at 200 gm for 6 weeks, then brought to 130 gm and held for 5 weeks; then brought back to 200 gm and held at this weight for 4 weeks.

Group 8 (RSA)—held at 200 gm for 6 weeks, brought to 130 gm and held for 5 weeks; then brought back to 200 gm and fed ad libitum for 4 weeks.

Group 9 (ad libitum controls)— fed ad libitum for the entire duration of the experiment.

These dietary patterns are shown diagrammatically in figure 1.

Analyses. All animals were anesthetized with pentobarbital sodium, and the

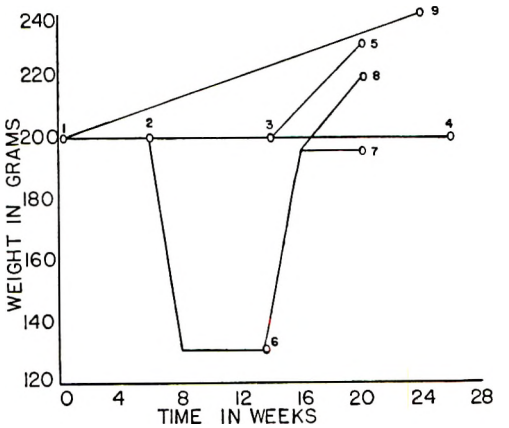

Fig. 1 Diagrammatic representation of weight patterns in animals in the various groups described in text.

liver removed. The rest of the animal, including food remaining in the digestive tract, was weighed. The carcass was dried to constant weight *in vacuo* at 50°C and the difference in weight before and after drying was recorded as body water. The dried carcass was then cut into small pieces and the fat extracted with ether for 12 hours in a Soxhlet apparatus. Body fat was calculated as the difference in carcass weight before and after ether extraction. The dried, fat-extracted carcass was covered with 400 ml of 20% sulfuric acid (reagent grade), filtered and the filtrate diluted to a standard volume with distilled water. Aliquots were taken for the determinations of nitrogen according to the Kjeldahl method described by Block and Weiss ('56).

Weighed portions of liver were ground in 95% ethyl alcohol. The suspension was then heated to 60°C for 30 minutes and the alcohol decanted. The tissue was extracted a second time with hot alcohol and then at room temperature with ether, each extraction lasting one-half hour. The final ether extract was filtered, all extracts combined, brought to standard volume, and aliquots taken for lipid determinations.

Total lipid was measured by a modification of the method of Bloor ('28). Total cholesterol was determined by the method of Sperry and Webb ('50). Phospholipid was measured by the method of Hawk and Bergheim ('37), with the phosphorus determined by the method of Sumner

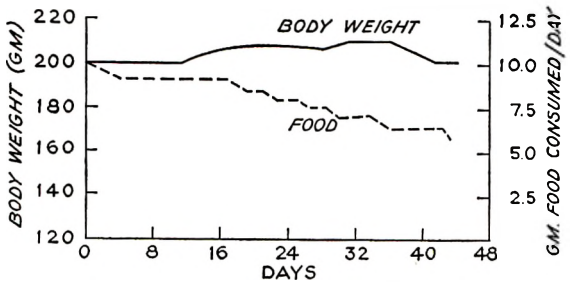

Fig. 2 Average body weights and daily food consumption for 41 rats fed the restricted diet for 44 days.

('44). A factor of 25 was used to convert phosphorus to phospholipid.

Experiment 2

All animals were killed by a sharp blow on the neck. Liver, heart, and kidneys were removed and trimmed of adhering fat. They were then weighed to the nearest 5 mg. Weights were converted to percentage of the total body weight.

All statistical computations were carried out using the "t" test for statistical significance as described in Snedecor ('46).

RESULTS

The relationship between food consumption and body weight during the first 44 days of caloric restriction is shown in figure 2. Average body weights (41 animals) varied between 199 and 211 gm, with a mean of 205 gm. During the same period of time food consumption declined from an initial value of 0.35 gm (1.45 Cal.) per gm of body weight per day to 0.23 gm (0.95 Cal.) per gm of body weight per day, a decrease of 35%. During the next 120 days of caloric restriction (5 animals of group 4) body weight remained between 190 and 210 gm, with a mean of 200 gm. Food consumption remained at 0.24 to 0.26 gm per gm of body weight per day. This is in agreement with the results of Quimby ('48) and Kaunitz et al. ('56).

In order to investigate the stability of the caloric-requirement-adaptation, the food consumption records of the animals in group 7 were examined. After adaptation to a daily food intake of 0.24 to 0.26 gm per gm body weight per day, these animals were starved until they had lost one third of their body weight. They were maintained at this weight for 5 weeks and then fed ad libitum until body weight returned to 200 gm. Caloric requirements were almost immediately re-established at 0.24 gm per gm of body weight per day and remained at this level for the next 4 weeks during which time body weight was held at 200 gm.

The results of body composition analyses for the various groups of animals are shown in table 2. The proportion of the total body weight that was represented by fat varied with the caloric intake. Statistical comparisons of the percentage of fat, however, indicate that differences between group 1 (zero-controls) and groups 2, 3, and 4 (the three restricted groups) were not significant at a 1% level. Furthermore, comparison of group 1 with group 7 shows that a period of restriction following starvation results in re-establishment of an amount of fat not statistically different from that of the control group or restricted groups. Ad libitum feeding, following either restriction or starvation, resulted in a significantly higher proportion of fat (1% level of significance). Group 9 (ad libitum controls) also had significantly more fat than group 1 animals. Differences between groups 5 (RRA) and 8 (RSA) and groups 4 (RRR) and 7 (RSR) were not significant. This would indicate that the final dietary periods used were of sufficient duration for the establishment of an equilibrium. That is, the final ad libitum periods or restricted periods were not dependent upon whether the animal had been starved or restricted during the preceding period.

The proportion of fat-free body weight that is represented by protein is also shown

TABLE 2

Body composition of rats fed indicated diets[1] (exp. 1)

Group	No. of animals	Diet[2]	Fat	Water	Protein
			% total body weight	*% fat-free weight*	*% fat-free weight*
1	10	Zero-time controls	6.3 ± 2.5[3]	71.6 ± 1.2	19.3 ± 0.1
2	6	R	4.0 ± 2.4	69.6 ± 0.8	18.9 ± 1.0
3	4	RR	3.8 ± 1.7	69.6 ± 2.0	19.1 ± 1.3
6	6	RS	1.8 ± 0.8	69.1 ± 2.0	20.1 ± 1.1
4	4	RRR	7.6 ± 3.5	68.8 ± 2.1	19.4 ± 0.4
5	3	RRA	11.8 ± 1.5	71.2 ± 2.3	19.0 ± 2.0
7	4	RSR	7.5 ± 1.8	70.9 ± 1.9	19.2 ± 0.7
8	4	RSA	12.1 ± 4.0	67.2 ± 0.2	20.0 ± 0.6
9	4	Ad libitum controls	16.5 ± 3.9	68.3 ± 1.4	21.3 ± 1.2

[1] See "Methods" for duration of diet periods.
[2] R, restricted to maintain 200 gm of weight; S, starved to 130 to 140 gm and held at that weight; A, refed ad libitum.
[3] Mean and standard deviation.

TABLE 3

Liver lipids of rats fed indicated diets[1] (exp. 1)

Group	No. of animals	Diet	Total lipid	Cholesterol	Phospholipid
1	3	Zero-time controls	3.57 ± 0.09[2]	0.24 ± 0.01	2.08 ± 0.41
2	4	R	4.18 ± 0.92	0.20 ± 0.02	2.21 ± 0.30
3	3	RR	3.66 ± 0.17	0.23 ± 0.03	2.51 ± 0.27
6	6	RS	3.90 ± 0.55	0.21 ± 0.03	2.41 ± 0.72
4	3	RRR	3.60 ± 0.31	0.18 ± 0.03	2.08 ± 0.12
5	4	RRA	3.80 ± 0.90	0.23 ± 0.10	2.64 ± 0.43
7	5	RSR	3.60 ± 0.83	0.18 ± 0.07	1.95 ± 0.31
8	4	RSA	3.50 ± 0.44	0.23 ± 0.03	2.09 ± 0.67
9	4	Ad libitum controls	3.50 ± 0.60	0.16 ± 0.02	1.93 ± 0.27

[1] All values are expressed as percentage of wet weight of liver.
[2] Mean and standard deviation.

in table 2. Statistical comparisons of groups 2 through 9 with group 1, and of group 5 (RRA) with 8 (RSA) and group 4 (RRR) with 7 (RSR) showed no significant differences, at a 1% level of confidence.

Body water, expressed as the percentage of fat-free body mass, showed significant changes at a 1% level. Group 2 (R) and 4 (RRR) differed significantly from group 1, although group 3 (RR) did not, and groups 8 (RSA) and 9 (ad libitum controls) differed significantly from group 1, although group 6 (RS) did not. No other significant differences were noted.

The percentages of total lipid, total cholesterol, and phospholipid in the livers of animals of the various groups are shown in table 3. Statistical comparison of liver lipid, cholesterol, and phospholipids of groups 2 through 9 with those of group 1 showed no significant differences (1% level of significance) in any of these categories.

In table 4 are shown the weights of liver, heart and both kidneys of the animals from experiment 2. These values are given as percentages of total body weight, with their standard deviations. No significant differences exist in heart and kidney weights between restricted animals and their controls when a 1% level of significance is used. Significant changes do seem to occur in liver weights, however. At the end of one week, restricted animals had smaller livers than their controls, but not significantly smaller than zero-time controls. After 8 weeks restricted animals had significantly larger livers than either the comparable control group or the zero-time controls.

DISCUSSION

Data presented here show, as do those of Quimby ('48) and Kaunitz et al. ('56), that caloric restriction to an extent which

TABLE 4

Organ weights of restricted and ad libitum-fed rats[1]

Group	No. weeks	Liver	Kidneys	Heart
Zero-time controls (15)[2]	0	3.60 ± 0.70[3]	0.89 ± 0.08	0.39 ± 0.05
Ad libitum-fed (9)	1	4.06 ± 0.77	0.84 ± 0.05	0.39 ± 0.04
Restricted (11)	1	3.17 ± 0.34	0.85 ± 0.08	0.37 ± 0.03
Ad libitum-fed (10)	2	3.28 ± 0.62	0.86 ± 0.08	0.39 ± 0.07
Restricted(10)	2	3.78 ± 0.81	0.84 ± 0.08	0.35 ± 0.04
Ad libitum-fed (8)	4	3.17 ± 0.31	0.82 ± 0.12	0.32 ± 0.03
Restricted (9)	4	3.29 ± 0.33	0.86 ± 0.09	0.36 ± 0.03
Ad libitum-fed (7)	6	3.64 ± 0.70	0.92 ± 0.05	0.35 ± 0.03
Restricted (5)	6	3.14 ± 0.28	0.84 ± 0.08	0.36 ± 0.05
Ad libitum-fed (6)	8	3.46 ± 0.28	0.82 ± 0.08	0.37 ± 0.06
Restricted (6)	8	4.40 ± 0.37[4]	0.91 ± 0.05	0.36 ± 0.03

[1] Organ weights are expressed as percentages of total body weights.
[2] Figures in parentheses indicate number of animals per group.
[3] Standard deviation.
[4] Underlined values differ from those of the comparable ad libitum groups by amounts significant at 1% level.

just maintains body weight is accompanied by some adjustment in caloric requirements. The decrease in caloric needs in response to this form of stress becomes established during the first 5 or 6 weeks of restriction, following which no further alteration in caloric requirement was produced by a period of starvation accompanied by weight loss. Whether the ad libitum feeding of group 5 (RRA) would re-establish the original pattern of food requirements has not been investigated.

The mechanism by which this adjustment takes place is not clear. Two possibilities were mentioned in the introduction (increased efficiency of absorption, and decreased basal metabolic rate). It has not been shown, however, that either of these would account for the changes observed.

It has been suggested (Keys and Brozek, '53; Shock, '54) that the basal metabolic rate is more closely related to the "active mass" of the body, namely, the total body mass less the stored fat and the weight of the skeleton, than to the total body mass—the weight of the total body with no exclusions. Although "fat-free body weight" is not synonymous with "active mass" it does exclude a less metabolically active compartment and, therefore, might be an indicator of energy requirements. Our data fail to show a significant change in fat-free body mass as a result of dietary restriction. Although the proportion of total body fat increased with weight on the ad libitum diets (groups 5, 8 and 9) it did not increase with age when total body weight was held constant (groups 2, 3, 4 and 7). The relative constancy of the water and protein moieties of the fat-free mass indicate that the adaptation to caloric restriction does not operate through changes in the composition of the lean-body mass. In the absence of actual measurements of basal metabolic rate during caloric restriction, our body composition data make it appear unlikely that this factor influences the caloric needs.

It was felt that the adaptation under investigation might involve changes in the metabolism and transport of lipids. Our measurements of liver total lipid, total cholesterol, and phospholipid, however, do not indicate that this is the case. These values are similar to those reported in the literature for control, or normal animals (Deuel, '55; Okey and Lyman, '56). That these values did not change during "starvation" or caloric restriction does not mean, of course, that alterations in lipid metabolism do not occur, but if they do, other procedures would be required to make them evident.

Data on organ weights indicate that the "caloric adaptation" does not make such metabolic demands that the heart and kidneys would hypertrophy. The reasons for the changes in liver weight are not clear and should perhaps be re-investigated. They do not show any readily explainable consistent changes.

The failure to demonstrate a relationship between caloric adaptation and either body composition, liver lipids, or organ size, suggests that the factors underlying the alteration in caloric requirements should be sought elsewhere. It should be pointed out, however, that there is no *a priori* reason to assume that a single factor is responsible for the entire change in caloric requirements. The change may be the result of several simultaneous alterations occurring in the animal: (1) an improved efficiency of absorption may make some contribution; (2) a decreased basal metabolic rate could also contribute to the decrease in caloric requirements, although the work of McCance and Mount ('60) does not support this; and (3) decreased total activity, if it occurs during caloric restriction, could be responsible for at least part of the decreased caloric requirements. We have not, however, observed any marked difference in activity between restricted and ad libitum fed animals. Enough change in activity to account for a 40% decrease in caloric requirements should be very apparent but a smaller change might occur and remain unnoticed. We are investigating this at the present time.

Furthermore, there also may be changes in the utilization of food, which could alter the energy available to the animal. The influence of caloric restriction on some aspects of carbohydrate metabolism is described in the succeeding paper.

SUMMARY

It has been confirmed that weight maintainance of rats by means of caloric restriction is accompanied by a gradual decline in caloric requirements. This caloric-restriction-adaptation was retained even after a period of severe caloric restriction accompanied by weight loss. Body composition, measured as the proportion of fat in the body, and protein in the fat-free body were the same following caloric restriction as in the zero-time control and ad libitum control animals. Some slight but significant changes were noted in body water. Liver total lipid, total cholesterol, and phospholipid levels were unaffected by caloric restriction. Although there were some significant changes in liver weight during caloric restriction, they did not follow any apparent pattern. Kidney and heart weights (as percentage of body weight) remained unchanged throughout the study. The constancy of body composition suggests that the caloric-restriction-adaptation operates through some compensatory mechanism such as alterations in total activity or through metabolic pathways which influence the efficiency of energy yields from the diet.

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Some Relationships Between Caloric Restriction and Body Weight in the Rat

II. THE METABOLISM OF RADIOACTIVE GLUCOSE AND THE ACTIVITY OF SOME TPN-LINKED ENZYMES IN THE LIVER[1]

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It was shown in the preceding paper (Lee and Lucia, '61) that, in the rat, restriction of caloric intake to a level which permits weight maintainance but not weight increase is accompanied by a gradual decrease in caloric requirements. This adaptation occurs during a period of 6 weeks following which caloric requirements are stabilized at about 60% of the initial needs. The caloric-restriction-adaptation is not accompanied by changes in gross body composition or the proportions of total lipid, cholesterol or phospholipid in the liver. The weight of the kidneys and the heart are not changed, but some changes are noted in the weight of the liver.

Since the caloric-restriction-adaptation was not explainable in terms of alterations in body composition, it was felt that some change in the manner of utilization of the dietary constitutents may have occurred. That is, this adaptation may cause, result from, or simply be accompanied by metabolic (or hormonal) changes which would alter the net utilizable energy extracted from the diet.

The manner in which carbohydrate is metabolized seemed worthy of investigation. There is ample evidence that the rat can metabolize glucose both by the hexose monophosphate pathway (HMP) and by the Embden-Meyerhof pathway (EMP) (Bloom et al., '53, '56; Muntz and Murphy, '57, and Murphy and Muntz, '57). Furthermore, it has been demonstrated that the relative proportions of glucose handled by these two pathways varies with the physiological or metabolic state of the animal. Although there may be some doubt concerning the caloric equivalence of glucose metabolized through these two pathways, it is conceivable that they do differ and that alterations in the relative activities of the two systems could account, at least in part, for the caloric-requirement-adaptation.

The effect of caloric restriction on the relative activities of the EMP and HMP was investigated by the use of glucose-1-C^{14} and glucose-6-C^{14}. The ratio of the amount of $C^{14}O_2$ recovered after administering glucose-1-C^{14} to the amount of $C^{14}O_2$ recovered after administering glucose-6-C^{14} (the C_1/C_6 ratio) was taken as a qualitative indicator of HMP activity. In addition, the *in vitro* activities of the two TPN-linked enzymes of the HMP were measured during the course of the caloric-restriction-adaptation.

Experiment 1. The animals used for body composition and liver lipid analyses described in the preceding paper (Lee and Lucia, '61) were used for this experiment. After a short period of fasting (several hours) each rat was given 2 μc of glucose-1-C^{14} [2] intraperitoneally, in 1 ml of isotonic saline. They were then placed in a metabolism chamber, and air was drawn

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[2] Nuclear Chicago Corporation.

through two 100-ml sodium hydroxide traps (in series). At the end of 0.5, 1.5, 2.5, and 3.5 hours the traps were replaced with fresh sodium hydroxide solutions. After 3.5 hours the animals were returned to their cages. Forty-eight to 72 hours later the procedure was repeated with the exception that glucose-6-C^{14} [3] was administered, instead of glucose-1-C^{14}. Samples were collected at the same time intervals. The animals were then sacrificed and the carcasses used for total body composition studies (Lee and Lucia, '61).

The sodium hydroxide solutions were analyzed for $C^{14}O_2$. An aliquot was distilled into *p*-(diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium hydroxide,[4] diluted with a solution containing 0.4% 2,5-diphenyloxazole[5] and 0.005% *p*-bis (2-(5-phenoyloxazolyl) 1-benzene[6] in toluene. The samples were analyzed in a Packard Tri-Carb Liquid Scintillation Counter. A correction was made for the background. The ratios of $C^{14}O_2$ from glucose-1-C^{14} and $C^{14}O_2$ from glucose-6-C^{14} was calculated from the total counts recovered in 3.5 hours.

Experiment 2. The animals used for organ weight analyses described in the preceding paper were also used for measurement of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. After the animals were sacrificed (as described previously) a 3-gm portion of liver was removed and homogenized in 6 ml of 0.25M sucrose. A glass Potter-type homogenizer with a loose-fitting Teflon pestle was used. Homogenization was carried out in an ice bath. The homogenate was lightly centrifuged (500 rpm, 5 min.) and the "supernatant" used for enzyme analyses.

The measurements of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were carried out as described by Fitch et al. ('59). The only alteration in the procedure was that 0.01 ml, instead of 0.02 ml, of homogenate was used where high enzyme activities were anticipated.

Protein analysis of the samples was carried out by the Biuret method of Kingsley ('42). Enzyme activities were calculated as micromoles of TPN reduced per minute per milligram of liver protein.

Statistical analysis was by the "t" test as described in Snedecor ('46).

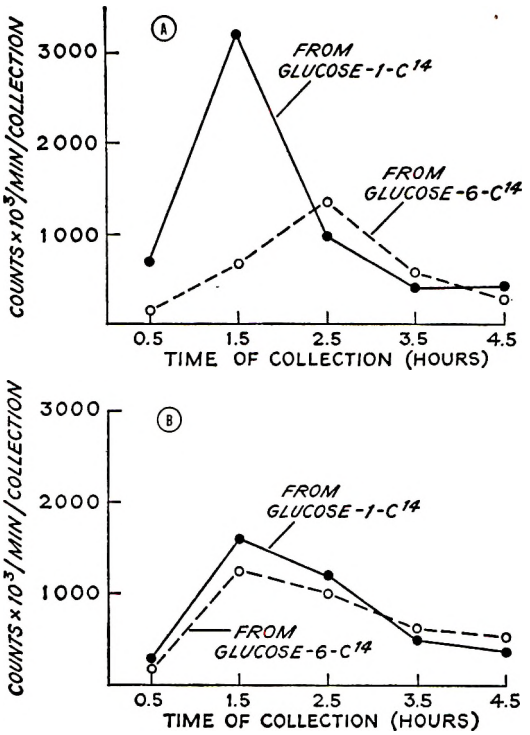

Fig. 1 Recovery of $C^{14}O_2$ from rats after the administration of glucose-1-C^{14} and glucose-6-C^{14}; A is from a rat in group 1 (controls not restricted); B is from an animal in group 6 (restricted and then starved).

RESULTS

Figure 1A shows the course of production of radioactive carbon dioxide in a control rat (no caloric restriction) given glucose-1-C^{14} and glucose-6-C^{14}. Figure 1B shows the course of production of radioactive carbon dioxide in an animal (similarly treated) from group 6 (RS). The ratio of $C^{14}O_2$ derived from glucose-1-C^{14} to the $C^{14}O_2$ derived from glucose-6-C^{14} in figure 1A is 1.87 and in figure 1B is 0.9. It may be seen in figure 1A that more $C^{14}O_2$ is obtained from glucose-1-C^{14} than from

[3] See footnote 2.

[4] Hydroxide of Hyamine, Packard Instrument Company.

[5] Purchased from Pilot Chemicals, Inc., Watertown, Massachusetts.

[6] See footnote 5.

TABLE 1

Recovery of $C^{14}O_2$ after administering glucose-1-C^{14} and glucose-6-C^{14} (counts × 10^3/10 min./sample)

Group	Animal	Glucose-1-C^{14}	Glucose-6-C^{14}	Ratio C_1/C_6	Ratio av.
1 Control	76	214	114	1.87	
	77	195	137	1.42	1.47
	69	164	148	1.11	
	Av.	191	133		
2 R	70	87	64	1.36	1.30
	67	78	63	1.24	
	Av.	83	64		
3 RR	26	128	105	1.17	1.15
	27	144	127	1.13	
	Av.	136	116		
5 RRA	35	169	133	1.27	1.04
	36	147	181	0.82	
	Av.	158	157		
6 RS	64	145	101	1.43	
	65	102	130	0.79	1.04
	66	134	149	0.90	
	Av.	127	127		
7 RSR	10	101	74	1.37	
	12	126	148	0.85	1.04
	13	107	120	0.90	
	Av.	111	114		
8 RSA	18	182	149	1.22	1.10
	19	122	125	0.98	
	Av.	152	137		
9 Ad libitum	88	186	139	1.37	1.39
	91	177	126	1.40	
	Av.	182	132		

glucose-6-C^{14} and the peak of the recovery from glucose-1-C^{14} precedes that from glucose-6-C^{14}. As shown in figure 1B, the amounts recovered and the position of peaks are almost identical.

In table 1 are shown the total recoveries of $C^{14}O_2$ following administration of glucose-1-C^{14} and glucose-6-C^{14} for each animal recorded by groups. Because of large variations within each group, individual results are recorded. The ratio of $C^{14}O_2$ from glucose-1-C^{14} to $C^{14}O_2$ from glucose-6-C^{14} decreases during the first two periods of caloric restriction (groups 2 and 3, as compared with group 1) and continues to decline in group 5 animals, although these animals have been refed ad libitum for 5 weeks. Animals which have undergone a period of severe caloric restriction associated with weight loss (groups 6 and 7) have average ratios approaching 1.0 and although a final period of ad libitum feeding (group 8) results in a ratio greater than 1.0, the two values contributing to this ratio do not differ much from the values for groups 6 and 7.

In table 2 are shown the results of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase measurements of liver homogenates of animals used for experiment 2. Standard deviations accompany each value in this table. Although the mean activity of 6-phosphogluconic acid dehydrogenase seems to increase slightly with the age of the animal, there is no marked difference between the ad libitum control and restricted animals in each group, nor is there any marked increase with time. On the other hand the glucose-6-phosphate dehydrogenase activity increases very greatly in the restricted animals, particularly during the first three

TABLE 2

Activities[1] of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in rat liver homogenates

Group	No. weeks	Glucose-6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase
Ad libitum-fed control (10)[2]	0	11.1 ± 3.9	5.9 ± 1.9
Restricted (9)	1	27.8 ± 5.3	6.9 ± 2.9
Ad libitum-fed (10)	1	15.2 ± 9.1	6.1 ± 2.6
Restricted (10)	2	36.5 ± 10.6	9.1 ± 2.6
Ad libitum-fed (10)	2	11.0 ± 6.5	8.1 ± 2.5
Restricted (8)	3	49.9 ± 11.8	9.5 ± 2.3
Ad libitum-fed (8)	3	12.3 ± 8.1	7.0 ± 2.8
Restricted (9)	4	43.3 ± 14.6	9.5 ± 2.3
Ad libitum-fed (9)	4	9.1 ± 4.6	9.1 ± 3.7
Restricted (6)	6	63.2 ± 11.0	12.3 ± 2.0
Ad libitum-fed (5)	6	17.2 ± 8.7	8.2 ± 3.1
Restricted (6)	8	60.5 ± 21.9	9.1 ± 2.2
Ad libitum-fed (6)	8	10.4 ± 9.7	8.7 ± 1.7

[1] Micromoles of TPN reduced/min./mg protein.
[2] Figures in parentheses indicate number of animals per group.

weeks. By the second week, the glucose-6-phosphate dehydrogenase activity of the restricted animals is significantly higher (1% level) than that of the comparable ad libitum group. By the third week the glucose-6-phosphate dehydrogenase activity of restricted animals is about 4 times that of ad libitum controls of the same age and remains high for the remainder of the 8 weeks. The enzyme activity of the ad libitum control animals remains at the same level, with only minor fluctuations for the entire 8 weeks.

DISCUSSION

The evidence for a non-Embden-Meyerhof glycolytic pathway in intact animals has been confusing. Bloom et al. ('53) were unable to demonstrate such a pathway in the intact rat but, from studies of liver slices, concluded that most glycolysis in the liver occurs via a direct oxidative pathway with the conversion of the first carbon of glucose to CO_2. Later Bloom et al. ('56) found evidence for a non-Embden-Meyerhof pathway in the intact rat by means of a modified technique. Muntz and Murphy ('57) and Murphy and Muntz ('57) demonstrated such a pathway in rat liver. Black et al. ('57) found similar evidence in the intact dairy cow. Demonstration of the hexose monophosphate pathway in the intact animal is complicated by several factors. For example, very active Embden-Meyerhof glycolysis in other tissues could mask direct oxidative glycolysis in the liver.

We feel, however, that our data on the utilization of glucose-1-C^{14} and glucose-6-C^{14} point to a measure of direct oxidation of glucose. Not only the greater production of $C^{14}O_2$ from glucose-1-C^{14} than from glucose-6-C^{14}, but also the earlier peak recovery of $C^{14}O_2$ from the former substrate than from the latter lends support to this conclusion.

Although only a few animals were examined and there was a large variation in $C^{14}O_2$ recoveries it appears that there is a measure of direct oxidation of glucose by the control rats and this diminishes during caloric restriction. The direct oxidation of glycolysis, however, did not entirely disappear by the end of 14 weeks, although the decline in caloric requirements for weight maintenance is observed in 6 weeks. A period of severe caloric restriction, resulting in a marked weight loss, is accompanied by a further decline in direct glucose oxidation. The metabolic alterations which occurred were not reversed to any significant extent by subsequent ad libitum feeding.

The data on ad libitum control animals (group 10) indicate that the decline in the activity of the hexose monophosphate pathway of metabolism is not merely a concomitant of aging in these animals.

The significance of the decrease in the direct oxidation of glucose during caloric restriction must be considered in relation to the mechanism of the caloric restriction adaptation. It has been demonstrated that no measurable net synthesis of fat

occurs during this time. It is possible, however, that the decrease in the production of TPN by the shunt mechanism leads to a diminished turnover of fat in the animal, although no net change in body fat occurs. The rate of turnover of body fat during caloric restriction remains to be investigated.

Although the hexose monophosphate pathway decreases during the caloric-restriction-adaptation this clearly does not result from a loss of enzyme activity. To the contrary, the *in vivo* activity decreases, but the glucose-6-phosphate dehydrogenase, as measured *in vitro,* rapidly increases. At the same time, the *in vitro* activity of 6-phosphogluconate dehydrogenase does not change significantly. The interpretation of this kind of data is difficult. It may well be that there is an increase in glucose-6-phosphate dehydrogenase in response to a decreased production of TPN. This postulated change in the amount of available TPN could come about through a decreased turnover of body fat, but in the absence of a change in the total amount of body fat. This hypothesis is now being tested.

Furthermore, the alterations in enzyme activity described in table 2 occur much more rapidly than does the alteration in caloric requirements during dietary restriction. The changes in the C_1/C_2 ratio, however, occur much more slowly than does the change in caloric requirements. Therefore, it is difficult to explain the caloric adaptation in terms of either of these observations.

It is possible that several other factors are involved in this adaptation. The relationships between caloric restriction and total activity, basal metabolic rate and lipid turnover are currently being examined in this laboratory.

SUMMARY

The relationship between caloric restriction (without concomitant weight loss) and glucose utilization has been investigated. Animals which have not been restricted show evidence of a direct oxidative pathway of glucose metabolism (hexose monophosphate pathway). Caloric restriction is accompanied by a decrease in the direct oxidation of glucose and when caloric restriction is sufficiently severe to cause weight loss the hexose monophosphate pathway disappears and is not re-established by a period of ad libitum feeding.

During caloric restriction the *in vitro* activity of 6-phosphogluconic acid dehydrogenase does not change but the activity of glucose-6-phosphate dehydrogenase increases about fourfold.

To what extent these changes are the underlying mechanisms of the caloric-restriction-adaptation is not clear but they may either cause or reflect a change in fat turnover and, thereby, an increase in the efficiency of food utilization.

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Factors Affecting the Absorbability of Saturated Fatty Acids in the Chick[1]

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Information on the absorbability of individual fatty acids and how it is affected by the presence of other fatty acids, neutral fat, fat breakdown products and by the form of the fatty acids is basic to an understanding of fat absorption. Since previous work from this laboratory has shown palmitic and stearic acids to be virtually unutilized by the chick when fed singly, the following experiments were undertaken: (1) comparison of the absorbability of the fatty acids of tallow, lard and soybean oil when fed in esterified form as mixed triglycerides with their absorbability when fed as mixtures of free fatty acids, and (2) determination of whether position in the triglyceride molecule affects absorbability of saturated fatty acids by studying the absorbability of palmitic acid in natural, partially rearranged and completely randomized lard.

1. *Absorbability of fatty acids in intact and hydrolyzed fats*

MATERIALS AND METHODS

Male crossbred (RIR × BPR) chicks were used in this experiment. Chicks were housed in electrically heated, thermostatically controlled battery brooders with raised wire-screen floors in a temperature-controlled laboratory. The chicks were reared to two weeks of age with reference diet E16 (Hill et al., '60), and then allotted on the basis of body weight to the experimental groups. Two duplicate groups of 10 chicks were fed each of the experimental diets from two to 4 weeks of age. Feed and water were supplied ad libitum.

The fat and fatty acid-containing diets were formulated by substituting 17.5 parts of tallow or tallow fatty acids, lard or lard fatty acids, soybean oil or soybean oil fatty acids, isocalorically for glucose in reference diet E16, assuming the metabolizable energy values of the three types of fat to be 6.9, 8.7 and 8.7 Cal. per gm, respectively, and using the predetermined value of 3.64 Cal. per gm for glucose.

The mixtures of fatty acids of tallow and lard were derived by hydrolysis from samples of the tallow and the lard which were studied as intact fats.[4] The following method of hydrolysis was used. Fat was hydrolyzed in a special, high-pressure autoclave with 0.25% ZnO catalyst for 1½ hours at a temperature of 480 to 520°F under a pressure of 600 to 700 psi. The fatty acids were acid-washed with 10% sulfuric acid solution to split the zinc soaps, settled and separated from the acid solution by decantation. They were then water-washed until the washings were neutral to methyl orange, whereupon they were vacuum-dried.

The fatty acid composition of the mixtures of fatty acids fed is given in table 1. These data are also applicable to the tallow and lard fed as intact fats since the starting materials were the same in each case. The soybean oil fed differed slightly in composition from the hydrolyzed soybean oil. The percentage composition of the soybean oil was palmitic, 10.6; stearic,

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[4] The hydrolyzed tallow and lard samples were prepared by the research laboratories of the Procter and Gamble Company, Cincinnati.

TABLE 1
Composition of fatty acid mixtures

	Percentage composition		
	Tallow fatty acids	Lard fatty acids	Soybean oil fatty acids
Myristic	3.4	1.3	0.2
Palmitic	25.1	24.8	19.2
Stearic	21.3	12.3	4.6
Myristoleic	1.6	—	—
Palmitoleic	8.4	4.8	—
Oleic	36.0	43.7	17.6
Linoleic	4.1	11.7	51.2
Linolenic	—	1.5	7.1

4.4; oleic, 23.5; linoleic, 52.8; and linolenic, 8.6.

Excreta were collected at 24-hour intervals on 4 successive days during the last week of the experimental period. Chromic oxide was incorporated in each of the diets as an index substance, eliminating the need for quantitative measurement of feed intake. The methods for processing excreta, conducting chemical determinations for chromic oxide and fat, and computing fat absorbability from these data have been described previously (Hill et al., '60; Renner and Hill, '60).

Fatty acid absorbability was determined from analysis of dietary and fecal fat for constituent fatty acids by gas chromatography. Prior to analysis, homogenized samples of the pooled excreta for each group were frozen in the wet state. Fecal fat was extracted by the method of Fowweather and Anderson ('46) and an aliquot used for the preparation of methyl esters using the microinteresterification method of Stoffel et al. ('59). The methyl esters were separated using a thermal conductivity type gas chromatograph, using diethyleneglycol succinate polymer as the partitioning agent.

Peaks were identified by comparing retention times of unknown components with retention times of a known mixture. Peak areas were computed by geometric approximation (peak height multiplied by width at half-peak height). The fraction by weight of a component in a mixture was computed according to the following formula proposed by Eastman ('57).

$$\frac{M_i}{W} = \frac{A_i \sqrt{M_i}}{\Sigma_i A_i \sqrt{M_i}}$$

where (M_i/W) is the fraction by weight of the ith component, A_i the area under the peak corresponding to the ith component, and M_i the molecular weight of the ith component. Orr and Callen[5] suggested its use for quantitative determinations of long-chain fatty acids.

Correction for endogenous fatty acids and fatty acids contributed by the constant diet ingredients was made from the low fat reference diet.

RESULTS AND DISCUSSION

Summarized in table 2 are data showing the absorbabilities of palmitic acid, stearic acid and the unsaturated fatty acids in tallow, lard and soybean oil when fed as the intact and hydrolyzed fat. The data show that palmitic and stearic acids were absorbed to 30 and 22%, respectively, when fed in a mixture with 50 parts of unsaturated fatty acids as in tallow fatty acids. When fed in a mixture with 62 parts of unsaturated fatty acids as in lard fatty acids, they were absorbed to 51 and 36%, respectively, whereas when present in a mixture with 76 parts of unsaturated fatty acids as in soybean oil fatty acids, the absorbability of palmitic and stearic acids increased to 84 and 78%, respectively. These data are in contrast with the observation that palmitic and stearic acids are virtually unutilized when fed singly and show that the absorbability of saturated fatty acids varies directly with the level of unsaturated fatty acids in the mixture.

The data also show that even greater absorbability of palmitic and stearic acid was obtained when they were fed in ester linkage as mixed triglycerides in the natural fats. The absorbability of palmitic and stearic acid in both tallow and lard was noted to be at least twice that of the respective fatty acid mixture. This increased absorbability of saturated fatty acids when fed in esterified form results in marked differences in over-all absorbability of intact and hydrolyzed fats. The data in table 2 show that over-all absorbability of the fatty acid mixture from tallow, lard or soybean oil to be in each case lower than the neutral fat from which it was derived.

[5] Orr, C. H., and J. E. Callen, 1959, personal communication.

TABLE 2

Absorbability of fatty acids in intact and hydrolyzed fats

	Absorbability, %			
	Palmitic acid	Stearic acid	Unsaturated fatty acids	Total lipid
Tallow	58	53	79	68
	57	53	83	70
	57[1]	*53*	*81*	*69*
Tallow fatty acids	28	23	75	51
	32	22	74	51
	30	*22*	*74*	*51*
Lard	94	74	94	92
	93	74	94	92
	94	*74*	*94*	*92*
Lard fatty acids	51	34	81	67
	51	38	81	68
	51	*36*	*81*	*68*
Soybean oil	93	92	98	97
	95	93	96	96
	94	*92*	*97*	*96*
Soybean oil fatty acids	86	82	92	90
	83	74	89	87
	84	*78*	*90*	*88*

[1] Italicized values are averages of duplicate lots; individual replicate values are in respective left columns.

This marked improvement in absorbability of saturated fatty acids when fed in esterified form supports the particulate theory of fat absorption in the chick and suggests that either neutral fat or some breakdown product of triglycerides is required for absorption of free fatty acids. It is also possible that having the saturated fatty acid in the glyceride form may be responsible for at least part of the improvement in absorbability; perhaps the extent to which they remain in this form determines the absorbability of the fat.

Several investigators, using rats as the experimental animal, have previously reported increased absorbability of saturated fatty acids when fed in esterified form. For example, calculations from the work of Mattil and Higgins ('45) indicate that the absorbability of stearic acid in distearomonoolein and monostearodiolein was 50 and 45%, respectively, when fed at 15% of the diet. Although these workers did not determine the absorbability of stearic acid when fed alone, an average value for the rat, obtained from a search of the literature, is 23%. More recently, Scribante and Favarger ('54) reported a threefold increase in absorbability of stearic acid when fed as distearomonoolein; fed alone at 15% of the diet stearic acid was 23% absorbed, as compared with 72% when fed as distearomonoolein.

The data in table 2 show further that palmitic acid in the triglycerides of lard was utilized to about the same extent as the unsaturated fatty acids. The high utilization of palmitic acid in lard triglycerides may be due to the fact that, according to Savary et al. ('57) and Mattson and Lutton ('58), lard contains a preponderance of its saturated fatty acids (palmitic acid) in the two-position. Since pancreatic lipase is specific for the primary ester linkages of triglycerides, it is possible that when palmitic acid is predominantly in the two-position, little free palmitic acid appears in the gastrointestinal tract. We have found that the absorbability of monopalmitin is much higher than the absorbability of free palmitic acid. Therefore the preponderance of palmitic acid in the two-position may account for the finding that palmitic acid in lard is 94% absorbed, and could be the determining factor in the high absorb-

ability of lard even though it contains 38% saturated fatty acids.

In order to test this hypothesis, an experiment was conducted to determine the absorbability of palmitic acid in samples of lard in which the fatty acids had been partially rearranged and completely randomized and should, therefore, show a gradation in the amount of palmitic acid located in the two-position.[6]

2. *Absorbability of palmitic acid in natural and rearranged lard*

MATERIALS AND METHODS

In this experiment, triplicate groups of 6 male crossbred (RIR × BPR) chicks were fed the experimental diets from one to 4 weeks of age. The lard-containing diets were formulated by substituting 20 parts of lard isocalorically for glucose in the semi-purified diet E17 which has been described in detail by Renner and Hill ('60). The methods of allotment, feeding and housing of the chicks and the collection of excreta and analyses of feed and feces were the same as in experiment 1.

RESULTS AND DISCUSSION

Summarized in table 3 are data showing the absorbability of the fatty acids in the three types of lard. Data show that absorbability of palmitic acid in natural, partially rearranged and completely randomized lard decreased with increasing randomization of the fatty acids in the constituent triglycerides. Analysis of variance (Snedecor, '56) and single degree of freedom comparisons showed that the differences in absorbability of palmitic acid were significant ($P < 0.05$). These results indicate that the absorbability of palmitic acid in lard varies with its position in the triglyceride molecule, the absorbability being higher when located in the two-position than in the one-position of lard triglycerides.

Similar statistical treatment of the absorbability data for stearic acid in the three types of lard showed that the absorbability of stearic acid remained unchanged. Since stearic acid is randomly distributed in the triglyceride of natural lard, rearrangement procedures would not be expected to alter absorbability.

Since palmitic acid makes up only 25% of the fatty acids of lard, a decrease of 15% in utilization of palmitic acid would decrease over-all fat absorbability by less than 4%, a difference which is difficult to detect. The over-all absorbability of natural, partially rearranged and completely randomized lard was found to be 92, 90 and 90% respectively.

[6] Samples of natural, partially rearranged and completely randomized lard were generously supplied by Dr. K. F. Mattil, Research Laboratories, Swift and Company, Chicago; his cooperation is gratefully acknowledged. Interesterification was conducted with sodium ethylate catalyst to achieve approximately 30% rearrangement for the partially rearranged sample, and complete randomization for the other.

TABLE 3

Absorbability of fatty acids in natural, partially rearranged and completely randomized lard

	Absorbability, %		
	Palmitic acid	Stearic acid	Unsaturated fatty acids
Natural lard	93	77	95
	92	76	95
	94	75	97
	93[1]	*76*	*96*
Partially rearranged lard	82	75	92
	83	80	95
	89	84	97
	85	*80*	*95*
Completely randomized lard	75	75	93
	80	80	95
	84	82	96
	80	*79*	*95*

[1] Italicized values are averages of triplicate lots; individual replicate values are in respective left columns.

SUMMARY

Absorbability by chicks of fatty acids in tallow, lard and soybean oil fed as the intact and hydrolyzed fats has been studied using gas chromatography. The absorbability of palmitic and stearic acid present in mixtures of unsaturated fatty acids increased as the level of unsaturated fatty acids in the mixture increased. Absorbability of palmitic and stearic acids in the fatty acid mixtures, however, was much less than their absorbability when fed in the form of mixed triglycerides present in the respective intact fats.

Additional studies on the absorbability by chicks of palmitic acid in natural, partially rearranged and completely randomized lard have shown that the absorbability of palmitic acid decreased with increasing randomization of the fatty acid in the constituent triglycerides. Thus, it appears that the absorbability of palmitic acid in lard varies with point of attachment of the acid in the triglyceride molecule, the absorbability being higher when located in the two-position than in the one-position of lard triglycerides.

Evidence obtained in the course of these experiments supports the *particulate theory* of fat absorption in the chick.

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Utilization of Fatty Acids by the Chicken[1]

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Information on the utilization of fatty acids by the chicken is extremely limited. Sunde ('56) obtained evidence that stearic acid was poorly absorbed by the chick, whereas oleic, linoleic and linolenic acids were well absorbed. Since no quantitative data were reported in this work, the following studies were undertaken to determine the utilization of lauric, myristic, palmitic, stearic and oleic acid by the chicken. Previous studies from this laboratory (Renner and Hill, '60) had shown that the ability of the chick to utilize tallow increases with age. Since it was possible that the utilization of fatty acids might also increase with age, utilization of fatty acids by the 4-week-old chick and the hen were determined in the studies to be reported.

Utilization of fatty acids was determined by two methods: (1) metabolizable energy determined by bomb calorimetry, and (2) absorbability. The purpose of using both methods was to determine whether the presence of fatty acids affected the utilization of other dietary constituents.

MATERIALS AND METHODS

Three experiments were run. To determine the utilization of oleic, stearic and palmitic acid, two duplicate lots of 10 male crossbred (RIR × BPR) chicks were fed the experimental diets from the age of two to 4 weeks; in the experiment to compare the utilization of lauric, myristic and palmitic acids, three lots of 6 male crossbred (RIR × BPR) chicks were fed each experimental diet from one to 4 weeks of age. The chicks were raised to one or two weeks of age with the low-fat reference diet and then distributed on the basis of body weight to the experimental lots. They were housed in electrically heated thermostatically controlled battery brooders with raised wire-screen floors in a temperature-controlled laboratory. Feed and water were supplied ad libitum, and collections of excreta were made during the 4th week of life.

In determining the utilization of fatty acids by the hen, Single Comb White Leghorns, 9 months old, were used. Three groups of two hens maintained in individual wire-floor layer cages were used for each dietary treatment. Collections of excreta were made after the experimental diets had been fed for one week.

Diets for the first experiment with chicks to study palmitic, stearic and oleic acids were formulated by substituting 20 parts of fatty acid for an equal weight of glucose[4] on a dry matter basis in the semi-purified reference diet E9 (Hill et al., '60). Diets for the second experiment containing lauric, myristic and palmitic acids were formulated in a similar manner using diet E17, the composition of which is given in table 1.

In formulating the fatty acid diets for hens, 20 parts of lauric, myristic, palmitic or stearic acid were substituted for an equal weight of sucrose in the low-fat reference diet E18 (table 1). The oleic acid diet was formulated by substituting oleic acid approximately isocalorically for sucrose using the values 8.31 and 3.79 Cal. per gm for oleic acid and sucrose, respectively.

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

TABLE 1
Composition of diets

	Diet E17	Diet E18
	%	%
Glucose[1]	50.7	—
Sucrose	—	52.4
Cellulose[2]	—	5.0
Soybean meal (50% protein)	35.0	32.0
Glycine	1.0	—
Methionine	0.5	0.1
Soybean oil	0.5	0.5
Fish solubles (dry basis)	1.0	—
Brewers' dried yeast	2.5	—
Dried whole whey	2.0	—
Mineral mixture	5.0[3]	7.9[4]
Vitamin mixture	0.5[3]	1.12[4]
Antioxidants	0.02[5]	0.01[6]
Chromic oxide "bread"[7]	1.0	1.0

[1] Cerelose, Corn Products Refining Company, New York.

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

[3] Mineral and vitamin mixtures supply in mg/100 gm of diet: $CaHPO_4$, 2630; $CaCO_3$, 1300; NaCl, 500; K_2HPO_4, 220; $MgSO_4$, 115; $FeSO_4 \cdot 7H_2O$, 28; ZnO, 6.3; $CuSO_4 \cdot 5H_2O$, 0.78; NaI, 0.26; Na_2SeO_3, 0.022; $MnSO_4$, 0.30; thiamine, 1.0; riboflavin, 1.0; Ca pantothenate, 4.0; biotin, 0.4; pyridoxine, 2.0; niacin, 8.0; folacin, 0.3; menadione, 0.3; vitamin B_{12}, 0.005; choline chloride, 300; vitamin A, 1000 U.S.P. units; vitamin D_3, 150 I.C. units; and vitamin E, 3.3 I.U.

[4] Mineral and vitamin mixtures supply in mg/100 gm of diet: $CaHPO_4$, 4000; $CaCO_3$, 3000; NaCl, 500; K_2HPO_4, 220; $MgSO_4$, 120; $FeSO_4 \cdot 7H_2O$, 20; $MnSO_4$, 15; ZnO, 6.0; $CuSO_4 \cdot 5H_2O$, 1.5; NaI, 0.3; Na_2SeO_3, 0.02; thiamine, 1.0; riboflavin, 2.0; Ca pantothenate, 5.0; niacin, 15.0; folacin, 0.2; pyridoxine·HCl, 1.5; biotin, 0.05; choline chloride, 120; vitamin B_{12}, 0.002; vitamin A, 100 U.S.P. units; vitamin D_3, 75 I.C. units; vitamin E, 11 I.U.

[5] Contains 0.01% DPPD and 0.01% BHT.

[6] Contains 0.01% BHT.

[7] Contains 30% Cr_2O_3 in wheat flour.

Chromic oxide was incorporated in each of the diets at a level of 0.3% as an index substance, in order to establish the amount of excreta produced per unit of diet intake without the need for quantitative measurement of either diet or excreta. The methods of processing excreta, conducting chemical analyses for nitrogen, combustible energy, chromic oxide and fecal fat, and computing metabolizable energy and fat absorbability from these data have been described previously (Hill and Anderson, '58; Hill et al., '60; Renner and Hill, '60). Moisture was determined by toluene distillation, AOAC ('55).

The samples of fatty acids used in this work were technical grade materials obtained through commercial channels. Data on their purity as determined in our laboratory by analysis by gas chromatography are given in table 2.

RESULTS AND DISCUSSION

Data on utilization of palmitic, stearic and oleic as determined by metabolizable energy and absorbability are summarized in table 3. These results show that palmitic and stearic acids were virtually unutilized by the chick at 4 weeks of age. This observation is in contrast with the range of values of 26 to 48% and 12 to 24% reported for the absorbability of palmitic and stearic acid in the rat by Cheng et al. ('49), Carroll ('58), Scribante and Favarger ('54) and Buensod and Favarger ('56). Other evidence that important species differences exist among monogastric animals is indicated by the finding of Lyman ('17) that palmitic acid is absorbed to the extent of 84% by the dog.

The data in table 3 also show that oleic acid was well utilized by the chick. This is in agreement with results reported for the rat by Carroll ('58) and Paul and McCay ('42), who found the absorbability of oleic acid to be 84 and 95%, respectively.

Similar data on utilization of lauric, myristic and palmitic acids by the chick

TABLE 2
Composition of fatty acids fed to chickens

Fatty acid fed	Percentage composition								
	C_{10}	C_{12}	C_{14}	$C_{14}{}^{1=}$	C_{16}	$C_{16}{}^{1=}$	C_{18}	$C_{18}{}^{1=}$	$C_{18}{}^{2=}$
Lauric	1	98	1	—	—	—	—	—	—
Myristic	—	2	97	—	1	—	—	—	—
Palmitic	—	—	3	—	89	2	6	—	—
Stearic	—	—	—	—	5	—	95	—	—
Oleic	—	—	3	2	5	9	2	74	5

are summarized in table 4, and show their absorbability to be 65, 25 and 5%, respectively. Carroll ('58) has reported the average absorbability of lauric, myristic and palmitic acid fed to young male rats at 10% of the diet to be 86, 64 and 39%, respectively. Thus, although chicks can utilize lauric and myristic acids to a greater extent than palmitic acid, their ability to utilize all three fatty acids is lower than that of the rat.

Several reasons can be proposed to explain the reason for the absorbability of saturated fatty acids being lower in chickens than in rats. First, the passage of food through the gastrointestinal tract of the chick is accomplished in approximately three hours as compared with 16 hours for the rat. Gidez and Karnovsky ('56) have suggested that when mucosal cells are loaded with fat, entry of further fat is prevented until that previously absorbed is further metabolized or is transported to other tissues; if this is true, then a slower passage through the intestine would favor greater absorption. Secondly, since according to Barnes and co-workers ('57) the rat consumes about 50% of its feces, it would seem that fatty acids might be more completely absorbed from the gastro-

TABLE 3

Utilization of individual fatty acids by the chick

Supplement to diet	Energy utilization		
	Metabolizable energy	Percentage of gross energy[1]	Absorbability
	Cal./gm	*%*	*%*
Palmitic acid	0.20	3	3
	−0.29	−3	−4
	−0.01[2]	*0*	*−1*
Stearic acid	0.16	2	3
	−0.90	−9	−7
	−0.37	*−4*	*−2*
Oleic acid	8.32	88	89
	8.30	88	88
	8.31	*88*	*88*

[1] Metabolizable energy as a percentage of gross energy of fatty acid.
[2] Italicized values are averages of duplicate lots; individual replicate values are in respective left columns.

TABLE 4

Utilization of individual fatty acids by the chick

Supplement to diet	Energy utilization		
	Metabolizable energy	Percentage of gross energy[1]	Absorbability
	Cal./gm	*%*	*%*
Lauric acid	6.18	70	67
	5.61	64	63
	5.72	65	66
	5.84[2]	66	65
Myristic acid	2.66	29	28
	1.71	19	23
	2.05	22	24
	2.14	23	25
Palmitic acid	0.30	3	3
	0.76	8	5
	0.81	9	7
	0.62	7	5

[1] Metabolizable energy as a percentage of gross energy of fatty acid.
[2] Italicized values are averages of duplicate lots; individual replicate values are in the respective left columns.

intestinal tract if permitted to recycle as the result of coprophagy.

Although chicks showed measurable utilization of lauric and myristic acids, their growth rate when fed diets containing these fatty acids was markedly reduced as shown by the data in table 5. An analysis of variance (Snedecor, '56) and application of Duncan's multiple range test (Federer, '55) showed that chicks fed lauric and myristic acids grew at a significantly slower rate than chicks fed the palmitic acid diet or the low-fat reference diet. Similar statistical treatment of the data on efficiency of gains showed that chicks fed lauric, myristic or palmitic acid utilized their diets similarly, but in each case, less efficiently than chicks fed the reference diet. Since the diets varied in the metabolizable energy supplied per gram, a more accurate estimate of efficiency is obtained by considering the metabolizable energy calories required per gram of gain. On this basis, the lauric acid diet was least well utilized and the myristic acid diet was significantly ($P < 0.05$) less well utilized than the palmitic acid diet or the reference diet were.

Autopsy of chicks at the completion of the experiment showed that the gizzard lining of chicks fed lauric acid was thickened, cornified and extremely rough. This condition was noted only in chicks receiving the lauric acid diet. It appeared that lauric acid had an irritating effect on at least part of the the gastrointestinal tract. Other physiological effects of lauric acid responsible for the marked depression in growth are at present unknown.

Data on the utilization of myristic, palmitic, stearic and oleic acids by the hen are summarized in table 6. Observations show that as chain length increased, absorbability of saturated fatty acids decreased. A similar inverse relationship was shown for the chick by the data in tables 2 and 3. Previously, Carroll ('58) reported similar findings for the rat.

Two hens receiving each diet containing myristic, stearic and oleic acid were removed from the experiment when it was noted that the diets were unacceptable to them. All 6 hens refused to eat the diet containing lauric acid and thus the utilization of lauric acid could not be determined. The complete refusal of hens to eat the lauric acid-containing diet was in contrast with the behavior of chicks, in which diet consumption was subnormal but sufficient for slow growth. The following modifications of the lauric acid-containing diet were unsuccessful in making it acceptable to the hens: (1) adding

TABLE 5

Rate and efficiency of growth of chicks fed diets containing fatty acids

Supplement to diet	Weight 4 weeks	Gm diet/ gm gain	M.E. Cal./ gm gain
	gm		
None (reference)	376	1.92	5.34
	413	1.92	5.20
	406	1.73	4.83
	398[1]	*1.86*	*5.12*
Lauric acid	215	2.18	7.05
	241	1.90	5.92
	207	2.27	7.12
	221	*2.12*	*6.69*
Myristic acid	327	2.15	5.51
	327	2.21	5.30
	316	2.28	5.61
	323	*2.21*	*5.47*
Palmitic acid	360	2.27	4.87
	389	2.16	4.82
	366	2.25	5.04
	371	*2.23*	*4.91*

[1] Italicized values are averages of triplicate lots; individual replicate values are in respective left columns.

TABLE 6
Utilization of fatty acids by the hen

Supplement to diet	No. hens	Energy utilization		
		Metabolizable energy	Percentage of gross energy[1]	Absorbability
		Cal./gm	*%*	*%*
Myristic acid	2	1.74	19	26
	1	3.11	34	39
	1	0.74	8	23
		1.86[2]	*20*	*29*
Palmitic acid	2	0.74	8	12
	2	0.94	10	14
	2	0.37	4	10
		0.68	*7*	*12*
Stearic acid	2	0.29	3	6
	2	−0.02	−0.2	2
		−0.14	*1*	*4*
Oleic acid	2	8.98	95	94
	2	9.16	97	94
		9.02	*96*	*94*

[1] Metabolizable energy as a percentage of gross energy of fatty acid.
[2] Italicized values are averages of duplicate or triplicate lots; individual replicate values are in the respective left columns.

an equimolar mixture of sodium and potassium bicarbonates at a level equal to one-half the molar concentration of lauric acid; (2) adding 1% of an antacid;[5] and (3) forming the diet into pellets.

Comparison of the absorbability data in tables 3, 4, 5 and 6 indicates that the ability of the hen to absorb fatty acids is 4 to 11% higher than that of the chick. Analysis of variance (Snedecor, '56) showed this difference to be significant ($P < 0.01$). The greater ability of the hen to utilize fatty acids helps to explain the earlier findings of Duckworth et al. ('50) and Renner and Hill ('60) that the ability of the chick to utilize mutton fat and beef tallow increases with age.

A comparison of the two methods of studying fatty acid utilization showed that metabolizable energy and absorbability gave generally similar results. The good agreement between the methods in the experiments with chicks showed that the presence of the high levels of fatty acids used did not adversely affect the ability of the chick to utilize other components of the diet. In the experiment with hens, however, the absorbability data showed somewhat higher utilization than did the metabolizable energy data for the saturated fatty acids, particularly myristic acid. Because of the variability evident in the data, no final conclusion can be drawn, but they suggest interference of these fatty acids with the utilization of other components of the diet under the conditions employed.

SUMMARY

1. Studies were conducted with chicks up to 4 weeks of age and with hens to determine the absorbability of single fatty acids fed at a level of 20% in a semi-purified diet. Absorbability was estimated both by determination of fecal lipids and by combustion analysis of energy utilization. The two methods were in generally close agreement.

2. In the chick, utilization of the saturated fatty acids from C_{12} to C_{18} decreased as chain length increased. Palmitic and stearic acids were essentially unutilized. Oleic acid was found to be approximately 88% utilized by the chick.

3. In the hen, utilization of the saturated fatty acids also decreased with increase in chain length. Absorbability of myristic, palmitic and stearic acids, however, was significantly greater for the hen than for the chick. Hens refused to con-

[5] Gelusil, a combination of magnesium trisilicate and aluminum hydroxide gel, Warner-Chilcott, Morris Plains, New Jersey.

sume a diet containing 20% of lauric acid.

4. Inclusion of lauric or myristic acid in the diets of chicks significantly reduced growth rate, the reduction being most marked for lauric acid.

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Cricket Nutrition

II. AN UNIDENTIFIED FACTOR IN THE NUTRITION OF ACHETA DOMESTICUS[1]

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In a study of comparative nutrition it was found that a diet which supported normal growth and reproduction through three generations of the german cockroach, *Blattella germanica* (L) (Luckey, '61) was inadequate for the house cricket, *Acheta domesticus* (L).[3] Supplementation of this syntype diet with powdered grass,[4] certain commercial liver fractions or yeast produced an increased growth rate. Ghouri ('58) reported that a purified diet which was adequate for cockroaches and stored product insects was inadequate for crickets. McFarlane et al. ('59) have shown that a mixture of B-vitamins can replace yeast in the purified diet using only the parameter of duration of the nymphal stage of male crickets. Growth of the crickets was faster, however, when they were fed the diet containing yeast. The growth stimulation effect of yeast is not confined to crickets; increased growth rates are usually found upon the addition of yeast to a synthetic or purified diet in the study of insect nutrition (Lipke and Fraenkel, '56). The fact that most of the diets used are considered to be adequate in known essential requirements indicates the existence of one or more unidentified growth factors in yeast for insects generally.

Reported herein is further evidence for the existence of a cricket growth factor(s), a survey of the potency of different source materials found to have stimulatory activity, the results of a survey of compounds for growth-promoting action, the identity of one factor found stimulatory to cricket growth, and the evidence for the existence of a second, as yet unidentified factor.

METHODS

Data obtained in the standardization of the methods used will be presented elsewhere.[5] One-day-old crickets, *A. domesticus* (L) hatched from eggs obtained from a stock colony were used throughout this investigation. In most of the experiments, crickets were fed the basal diet for one week prior to assay. This procedure greatly improved the reliability of the assay as many crickets die for various reasons during the first few days after hatching regardless of the diet given them. At one week of age 6 crickets (each weighing approximately 1.5 mg) were selected at random and placed in a battery jar ($10 \times 10 \times 10$ cm) containing about 2 gm of the diet and a cotton stoppered Erlenmeyer flask containing distilled water (fig. 1). Two jars (12 crickets) were used for each diet in each experiment. The battery jars were covered with cheese cloth and placed in an incubator maintained at $37 \pm 1°C$ and $58 \pm 2\%$ relative humidity for the period of the assay. At the end of this period the crickets were individually weighed, their average weight determined and the data analyzed statistically. The

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[2] Department of Entomology, College of Agriculture, University of Missouri, Columbia, Missouri.
[3] Luckey, T. D., and P. C. Stone 1961 Manuscript in preparation.
[4] The grass used was Cerophyll, a mixture of processed, tender young blades of rye, wheat, oats and cocksfoot obtained from the Cerophyll Company, Kansas City, Missouri.
[5] Stone, P. C., P. F. Neville and T. D. Luckey 1961 Manuscript in preparation.

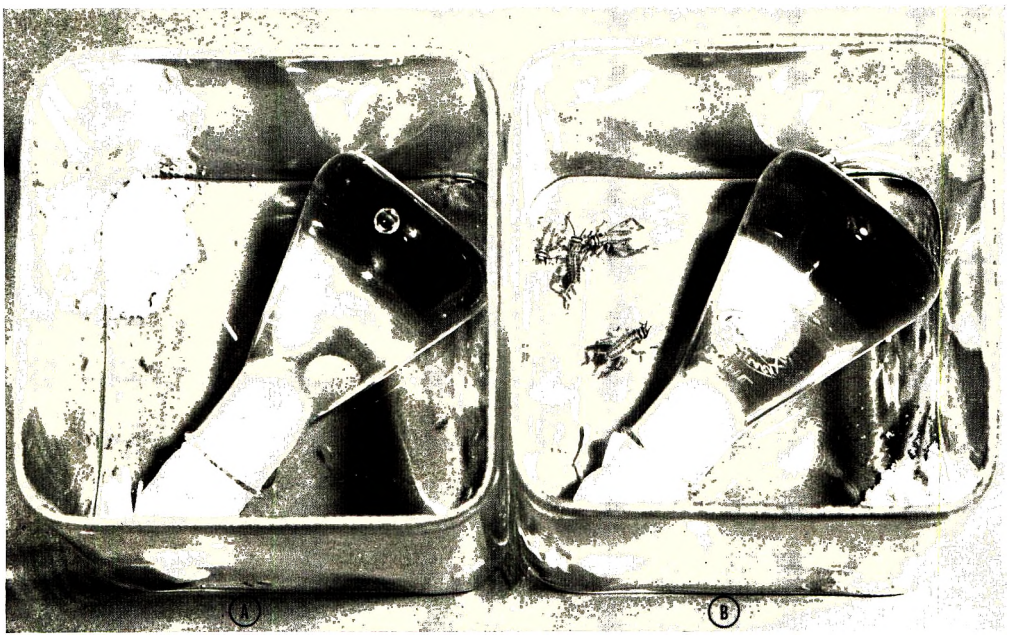

Fig. 1 Assay containers for crickets; (A) one-week-old crickets, (B) three-week-old crickets.

average weight at 21 days of age was the only index of activity used. A few experiments were run for 30 days. Either period was adequate for a good assay and yet short enough to eliminate the variability due to sex. Weight and length were found to give similar data. Survival was usually 90 to 100%.

The statistic used was an analysis of variance of disproportionate sub-class numbers (Snedecor, '50; Hawk et al., '47) to determine the least significant difference (LSD) of the means at the 5 and 1% level of probability. The LSD as reported in this paper is biased slightly by dividing by the average number of survivors minus one rather than the average:

$$\text{LSD} = t \times \sqrt{\frac{2 \times \text{Error mean squared}}{(\text{Av. number survivors} - 1)}}$$

This was done in order to produce a larger LSD and to lessen the possibility of accepting a false significant difference between two mean weights of crickets. The LSD obtained from this analysis of variance means that any two mean weights of crickets within a given experiment having a difference equal to or greater than the LSD are different at the given level of significance.

The compositions of the basal diets are given in table 1 and the sequence relating to their development is given in the results section. The separate dry ingredients, with the exception of the vitamins and cholesterol, were ground in a ball mill until they would pass through a 200-mesh sieve. After sifting, the dry ingredients were weighed and mixed in a ball mill for an additional 6 hours. Then the corn oil, fat-soluble vitamins and cholesterol were added along with enough ether to produce a slurry and the entire diet was mixed with an electric mixer until the ether had evaporated completely. The materials to be assayed were added to this basal diet and mixed together for an additional 15 minutes by electric mixer. If the assay material happened to be lipid, ether was used to aid the mixing in the same manner as described.

Although it was desirable to maintain a constant basal diet, certain changes were considered necessary and were made during the course of the investigation.[6] These changes were: (1) an increase in the cholesterol content of the diet (C series); (2) the elimination of vitamins C and D

[6] See footnote 5.

TABLE 1

Composition of basal diets

	Diet			
	U	C[1]	MC	HMC[2]
	%	%	%	%
Casein	30.0	30.0	30.0	30.0
Corn oil	8.0	8.0	8.0	8.0
Corn starch	30.0	30.0	30.0	30.0
Cellulose	12.0	12.0	12.0	12.0
Sucrose	12.0	12.0	12.0	12.0
Total salts[3]	8.0	8.0	8.0	8.0
Ascorbic acid	1.0	1.0		
Vitamin A (10,000 I.U. per kg)	+	+	+	+
Vitamin D_3 (2000 I.U. per kg)	+	+		
α-Tocopherol	0.01	0.01	0.01	0.01
Vitamin K_3 (menadione)	0.001	0.001	0.001	0.001
B-vitamin mix[4]	0.423	0.423	0.423	0.423
Cholesterol		0.1–0.4	0.4	0.4

[1] C-1 diet contains 0.1% cholesterol; C-2 diet, 0.2% cholesterol; C-4 diet, 0.4% cholesterol.
[2] HMC-80 refers to an 80-mesh diet; HMC-200 refers to a 200-mesh diet.
[3] Total salts (gm/kg): K acetate, 20.0; $CaCO_3$, 18.0; Na_2HPO_4, 12.0; $CaHPO_4$, 13.5; NaCl, 3.0; KI, 0.045; $MgSO_4 \cdot 7H_2O$, 4.5; MgO, 4.0; $MnSO_4 \cdot 4H_2O$, 0.75; $Fe(C_6H_5O_7)_2$, 4.5; $CuSO_4 \cdot 5H_2O$, 0.23; $CoCl \cdot 6H_2O$, 0.03; $NaMoO_4 \cdot 2H_2O$, 0.30; $ZnSO_4 \cdot 7H_2O$, 0.06; $Na_2B_4O_7 \cdot 10H_2O$, 0.03; $KAl(SO_4) \cdot 10H_2O$, 0.045.
[4] B-vitamin mix (gm/kg): thiamine·HCl, 0.02; nicotinamide, 0.1; Ca-*d*-pantothenate, 0.05; inositol, 2.0; choline·HCl, 2.0; pyridoxine·HCl, 0.02; biotin, 0.01; folic acid, 0.02; vitamin B_{12}, 0.00005.

TABLE 2

Comparison of growth-promoting properties of various source materials with respect to different basal diets

Source materials	Basal diets						
	C-1	C-2	C-4	MC	HMC	HMC-80	HMC-200
Yeast (3% or more)	0[1]	++[2]	++	++	++	++	++
Liver fraction "A"	++	++	0	0			0
Kidney powder (3% or more)	++	++	++	++			++
Dried whole beef kidney		+[3]		+			
Whole liver concentrate	0	+	0				
Royal jelly	0		++				
Kidney powder 70 (5% or more)				+			

[1] 0 Indicates material was tested and found ineffective.
[2] ++ Significant increase (P of 0.01 or less) in average weight.
[3] + Significant increase (P between 0.05 and 0.01) in average weight.

(MC diet); and (3) a decrease in the particle size of the diet (HMC-80-200).

The method used to ash grass and yeast was that described by Lepper (AOAC, '50).

RESULTS

Of the two materials, grass and yeast,[7] first found to have a growth-stimulating effect on crickets, yeast was consistently the more potent. A survey of many natural products representing plant, animal and bacterial materials was made and these materials were compared with yeast in order to begin fractionation of the most potent source. Those materials producing a significant increase in the growth of crickets are shown in table 2. Yeast, kidney powder[8] (desiccated and defatted at 40°C) and dried whole beef kidney produced significant increases in the growth of crickets with all of the base diets to which they were added. Hog kidney powder-70[9] (desiccated and defatted at 70°C) had approximately one-half of the growth

[7] "Yeast" refers to dried Torula yeast, type B obtained from the Lake States Yeast Corporation, Rhinelander, Wisconsin.
[8] "Kidney powder" refers to a pork kidney powder desiccated and defatted at 40°C obtained from the Viobin Corporation, Monticello, Illinois.
[9] "Kidney Powder-70" refers to a pork kidney powder desiccated and defatted at 70° obtained from the Viobin Corporation, Monticello, Illinois.

TABLE 3

Sources of growth factor and their effects with different basal diets

Description of diet	No. survivors	Average weight
		mg
LSD 1% 9.5 mg; 5% 7.1 mg		
C-1, Control	7	3.0
C-1 + 3% Yeast	8	8.6
C-1 + 3% Liver fraction "A"	7	15.3
C-1 + 3% Kidney powder	9	24.2
C-1 + 3% Royal jelly	7	3.2
LSD 1% 6.7 mg; 5% 5.0 mg		
C-2, Control	10	9.1
C-2 + 3% Yeast	10	17.3
C-2 + 3% Liver fraction "A"	9	20.2
C-2 + 3% Kidney powder	10	22.8
LSD 1% 9.5 mg; 5% 7.1 mg		
C-4, Control	10	10.4
C-4 + 3% Yeast	8	19.1
C-4 + 3% Liver fraction "A"	9	17.4
C-4 + 3% Kidney powder	10	18.7
C-4 + 3% Royal jelly	9	22.9
LSD 1% 8.7 mg		
MC, Control	7	6.7
MC + 3% Yeast	10	27.4
MC + 3% Kidney powder	6	20.2
MC + 3% Liver fraction "A"	9	8.6

stimulating activity of the kidney powder, indicating that the factor was either destroyed because of the higher temperature at which the former was prepared or was more soluble in the lipid solvent at the higher temperature and would have been found in the lipid portion.

The activities of royal jelly[10] and liver fraction "A"[11] appeared to be dependent upon the cholesterol content of the base diet but in a manner opposite to each other (table 3). Royal jelly was quite stimulatory when the diet contained 0.4% of cholesterol (C-4 diet) but showed no activity at all when fed with the C-1 basal diet (0.1% of cholesterol). Liver fraction "A" activity, however, diminished as the cholesterol content of the basal diet was increased. With basal diets C-1 and C-2 there was a 1% significant difference between the basal diets and the liver fraction "A" containing diets but with diets C-4 and MC (containing 0.4% of cholesterol and no vitamin C or D) this significant difference no longer existed. A Lieberman-Burchard (Hawk et al., '47) test conducted on the kidney powder, yeast and liver fraction "A" was positive for the last material, indicating that at least part of the growth stimulation observed in the early work was due to cholesterol or a similarly reacting sterol present in liver fraction "A." Royal jelly was dropped as a source material at this point in the investigation because it was no more stimulatory than yeast or kidney powder and because the latter two materials were more readily available.

Growth curves for kidney powder, yeast and liver fraction "A" are presented in figure 2. These curves indicate that 3% of kidney powder and 4% of yeast produce near-optimum growth in the crickets. The data in figure 2 also illustrates well the lack of stimulatory activity in liver fraction "A" even when it was added to the diet to the extent of 21%.

Attempts were made to fractionate yeast and thereby isolate the growth factor after it was determined that diets containing the ash of yeast or rye grass or double B-vitamins produced no better growth in the crickets than did the control diets.

[10] Royal Jelly obtained from the Prairie View Honey Company, Detroit.

[11] "Liver Fraction-A" obtained from Wilson and Company, Chicago.

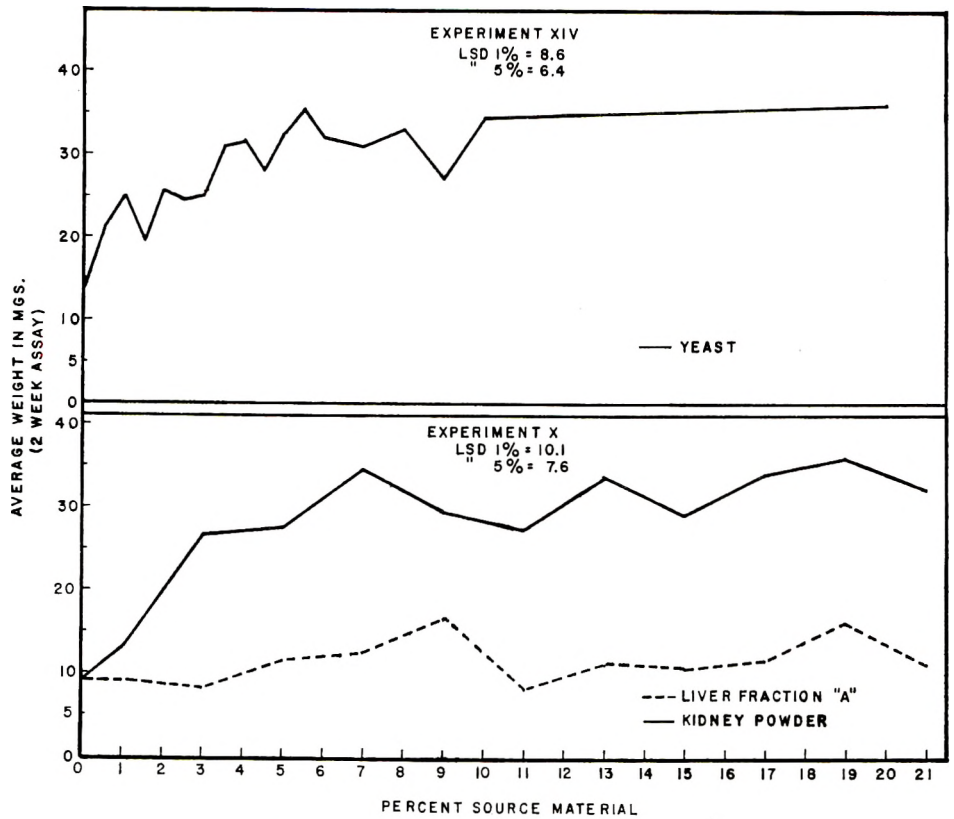

Fig. 2 Source material growth curves.

At the same time that the experiments with the yeast fractionation were being carried out, various compounds were being assayed for possible growth-promoting activity. Those compounds which apparently had no effect on the crickets' growth rate are: 2% of an equal mixture of cytosine, guanine, uracil, thymine, adenine or cytidine, uridine, adenosine, guanosine, thymidine or uridylic acid, cytidylic acid, guanylic acid, adenylic acid; 2% of an equal mixture of RNA and DNA; 0.1, 0.3 and 0.5% of squalene or mevalonic acid; 0.1% of β-sitosterol, stigmasterol or ergosterol; 0.1% of carnitine; 0.2% *p*-aminobenzoic acid; 0.1 and 0.4% of levulose, lactose, mannose, mannitol or glucose; 5% of gelatin; 0.4 and 1.0% of glycine and/or cystine; 0.45% of glutathione; 1% of glycine, 0.8% of DL-methionine and 0.8% of arginine; 1% of glycine and 0.8% of DL-methionine; 1% of glycine and 0.8% of cystine; 0.45 and 1.8% of DL-methionine; 0.96 and 3.84% of DL-tryptophan; 0.6 and 1.2% of ornithine; 0.6 and 1.2% of urea; 1.2% of urea and ornithine; 0.6 and 1.2% of glutamate (Na); 1.2% of glutamate and 1% of aspartate; 0.75 and 3% of histidine; 0.45 and 1.8% of DL-isoleucine; 0.27 and 0.82% of guanidoacetic acid; 0.34 and 1% of creatine; 0.27 and 0.82% of creatinine; 0.5 and 1% of hydroxyproline; 0.14 and 0.41% of guanidine; and 0.5% of NH_4PO_4. Among the ineffective compounds are *p*-aminobenzoic acid, carnitine, and purines and pyrimidines and their derivatives.

A significant growth stimulation was obtained through the use of lactalbumin and trypsin, which suggested a lack of amino acids (table 4). Of the amino acids used to fortify the basal diets, only a few altered the growth rate of the crickets. DL-Methionine, when fed at a concentration of 1.8% of the basal diet, significantly

inhibited the growth of the crickets as compared with the basal diet, although at a concentration of 0.45%, no difference from the basal diet was observed. Whether these effects are due to amino acid imbalance or an inability to utilize the D-form of methionine remains to be determined. However, 1.8% of DL-methionine did not inhibit growth when fed with L-arginine and DL-tryptophan. Diets containing additional L-arginine only and with DL-methionine and DL-tryptophan produced highly significant results (table 4). These experiments showed that diets supplemented with L-arginine produced an average weight in crickets comparable to those containing 4% of yeast. The results of studying the quantitative requirements for arginine are presented in figure 3. There are two peaks on both curves where optimum growth is obtained; one at 0.3 to 0.5% and the other at 1.2% of arginine and above. The growth rate of the crickets was not increased by the addition of 1% of yeast to diets containing these amounts of arginine. Cricket growth, however, was greatly stimulated by the additional 1% of yeast to the diets between these two points. The double peaks in these two growth curves suggest the hormoligosis effect observed in studying the sodium requirement of crickets by Luckey and Stone ('60).

Further evidence that 1.2% of arginine replaces the growth factor is provided by the results, shown in table 5, in which the various source materials were assayed alone and with an additional 1.2% of arginine. There was no significant difference between the average weights of crickets fed diets fortified with kidney powder or yeast with and without arginine.

TABLE 4

Compounds affecting cricket growth

Experiment	Compounds added to diet	HMC-80		HMC-200	
		(LSD) 1%	5%	1%	5%
17	Lactalbumin in place of casein		+		
	5% Lactalbumin		+		
	Total protein, 10% casein, 20% lactalbumin	+			
	1% Glycine, 0.8% methionine, 0.8% arginine	+			
18	2% Trypsin		+		
21	0.1% Mannitol				+
22	1.8% DL-Methionine				−
	0.3% L-Arginine				+
	1.2% L-Arginine			+	
	0.45% DL-Methionine, 0.3% L-arginine 0.96% DL-tryptophan			+	
28	1.2% DL-Citrulline				+
29	0.6% L-Arginine, 0.6% DL-citrulline				+

TABLE 5

Effects of L-arginine and/or source materials on cricket growth

Diets (from experiment 26, table 1) LSD 5% = 7.4, 1% = 9.8	Survivors[1]	Average weight
		mg
HMC-200	11	20.1
HMC-200 + 1% Yeast	10	27.2
HMC-200 + 4% Yeast	11	31.1
HMC-200 + 1.2% L-Arginine	10	34.7
HMC-200 + 4% Yeast, 1.2% L-arginine	11	33.3
HMC-200 + 4% Kidney powder	10	34.7
HMC-200 + 4% Kidney powder, 1.2% L-arginine	11	38.4

[1] Three-week assay: started 12 one-day-old crickets/diet.

TABLE 6
Dry weights of crickets

Diet	Survivors[1]	Average weight	Average % dry weight	Average % water
		mg		
HMC-200	10	22.2	27.8	72.2
HMC-200+1% Yeast	23	27.9	26.4	73.6
HMC-200+4% Yeast	18	40.2	25.9	74.1
HMC-200+1.2% Arginine	21	29.7	26.2	73.8
HMC-200+0.6% Arginine, 0.6% DL-citrulline	23	28.7	26.5	73.5
HMC-200+0.6% L-Arginine and 0.9% citrulline	22	22.0	25.6	74.4

[1] Three-week assay: started 24 one-day-old crickets/diet.

Because of the stimulation of growth resulting from the addition of high levels of arginine to the diet, various compounds associated directly or indirectly with the "urea cycle" and arginine were tested. DL-Citrulline was found to stimulate cricket growth (table 4) almost as well as arginine, when added to the diet alone or in combination with arginine. Ornithine, however, had no effect on the growth rate.

The increase in weight observed in these experiments was not due to an increase in the water content of the cricket. A determination of the percentage dry weight of crickets (table 6) reared with several diets shows that even though the average weights of the crickets receiving supplemented diets were twice the average of the control diet group, the percentage dry weight was constant for all.

DISCUSSION

The first growth-limiting factor found in these studies was cholesterol. Experiments conducted to develop the best basal diet had indicated that the C-1 and C-2 diets contained too low a concentration of cholesterol to produce optimum growth in the cricket; therefore, the concentration was increased to 0.4% in all succeeding diets.[12] Liver fraction "A," one of the early source materials, was eliminated as such when the cholesterol content of the diet was increased from 0.1 or 0.2 to 0.4%. This increase in cholesterol did not lessen the increase in growth resulting from kidney powder, dried whole beef kidney, yeast or royal jelly, indicating that at least one more growth-stimulating factor was present.

Arginine was a second limiting factor under the conditions used in these experiments. The casein[13] (approximately 88% protein) contributed approximately 1.2% of arginine (Stokes et al., '45) to the entire diet. The yeast, on the other hand, which is 47% protein[14] should contain approximately 2.5% of arginine, based on values of 4.5% of arginine and 16% of nitrogen (Stokes et al., '45). Therefore the addition of 4% of yeast to a diet should contribute only about 0.1 to 0.2% of arginine to the entire diet; and this, if one assumes that yeast contains some citrulline (no values could be found for the citrulline content of yeast) as well, could account for the increased growth obtained by supplementing the diet with 0.3 to 0.5% of arginine. The small amount of arginine and citrulline added to the diet by 4% of yeast, however, cannot account for the increased growth rate that resulted from supplementing the diet with 1.2% of pure arginine (the arginine concentration giving the most consistent results) because for yeast to supply this amount one would have to add approximately 48% of yeast to the diet. Whatever the factor may be in yeast, it would appear that it is something for which high levels of arginine and/or citrulline can substitute. The total amount of arginine in the 1.2% supplemented diet should be about 2.4% which is similar to the arginine requirement reported for chicks fed a high casein diet by Savage and O'Dell ('60). Apparently an amino acid imbalance is present. The lack of growth stimulation in the presence of 0.8 and 2.0%

[12] See footnote 5.

[13] The casein used was obtained from the Sheffield Chemical Company, Inc., Norwich, New York.

[14] See footnote 7.

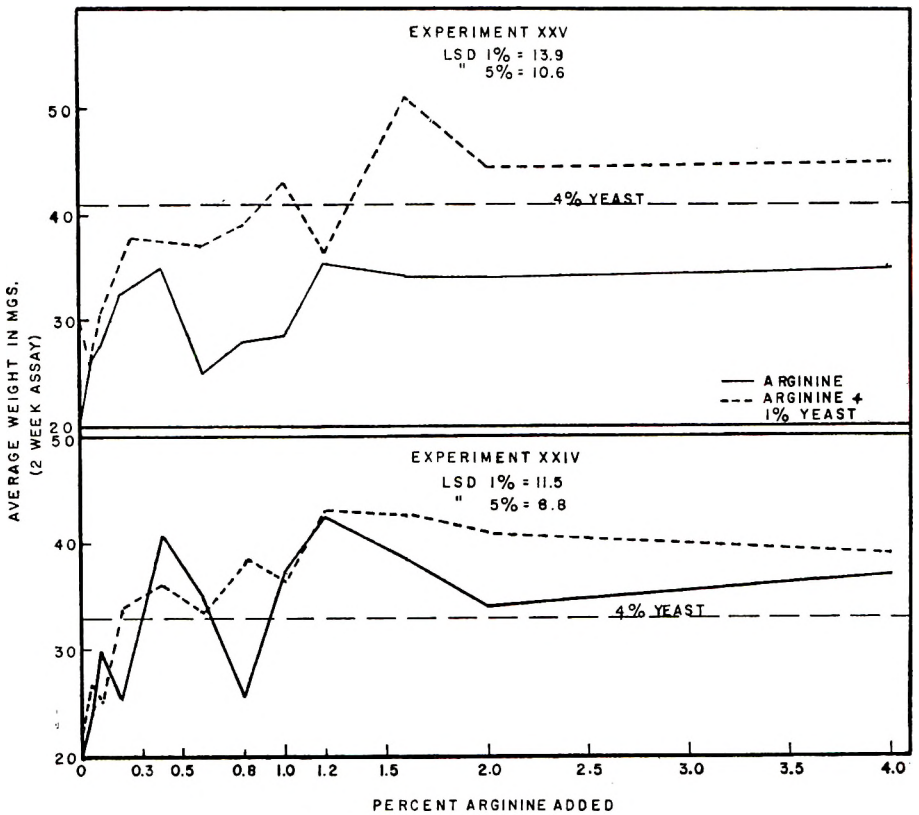

Fig. 3 Arginine growth curves.

arginine, as seen in figure 3, indicates the possibility of a third factor besides arginine and citrulline. Such a growth depression does not occur when the natural materials are added to the diet.

Because it is generally assumed that uric acid and not urea is the primary nitrogen excretion product in insects (Florkin, '49) it is interesting to speculate on the need of the cricket for so much arginine. If a functioning "urea cycle" was present, then arginine, citrulline and ornithine should be interchangeable. The fact that arginine and citrulline were active and ornithine was not, makes it improbable that such a cycle exists in the cricket. These results agree with those obtained by feeding the same amino acids to *Drosophila melanogaster* (Taylor, '56). Apparently the stimulatory effect of arginine is not due only to the amidine or guanido group since guanidoacetic acid and guanidine were ineffective when added to the base diet. Possibly the explanation for this high arginine requirement of the cricket is connected with the role arginine plays as the precursor of phosphoarginine, the phosphogen of insects, or as the precursor of γ-guanidobutyric acid and δ-guanido-α-ketovaleric acid, the guanidine derivatives considered to be characteristic of insects (Garica et al., '56).

SUMMARY

Liver fraction "A," kidney powder, dried Torula yeast and royal jelly, when added in small quantities to a purified diet which was adequate for a variety of other species, have been shown to greatly stimulate the growth rate of crickets, *Acheta domesticus*. Through this investigation, two growth factors have been identified and evidence for the existence of another factor or factors has been obtained.

The results obtained have shown that the ash of grass and yeast, carnitine,

purines and pyrimidines and their derivatives, several sugars, higher levels of B-vitamins and some amino acids, as well as many fractions of yeast were ineffective as growth stimulators. The stimulation obtained with liver fraction "A" was eliminated by increasing the cholesterol content of the purified diet. This indicated that one of the factors was cholesterol or a related sterol.

Cricket growth equivalent to that produced by the yeast and kidney powder was achieved by the addition of high levels of L-arginine to the diet. It was also observed that citrulline, but not ornithine, would stimulate growth indicating the absence of a functioning "urea cycle" in crickets.

Although arginine produced growth equivalent to that produced by the source materials, the quantity required to do so suggests that it is not the only factor present in yeast and kidney powder. The evidence indicates that there is another factor or factors.

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Disproportionate Growth in Offspring of Manganese-Deficient Rats

I. THE LONG BONES[1]

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Studies of maganese deficiency in a number of species have demonstrated the importance of this trace element for normal skeletal development (see reviews by Underwood, '56; Cotzias, '58). In chicks, manganese deficiency results in perosis, characterized by enlargement and malformation of the tibio-metatarsal joint (Wilgus et al., '37). Rabbits given manganese-deficient rations show severely deformed front legs, shortening and bowing of the bones, and decreased ash content (Smith et al., '44). Similar observations have been made in swine given a manganese-deficient diet from weaning (Plumlee et al., '56).

The importance of manganese during the *embryonic* period for normal development of the skeleton is shown by the work of Lyons and Insko ('37) who observed chondrodystrophy in chick embryos from manganese-deficient hens. This condition, characterized by shortening of the legs, wings, and lower mandible, and a globular contour of the head, was prevented by supplementation with manganese. The observation of Caskey et al. ('39) of micromelia in newly-hatched chicks from manganese-deficient hens, and the apparently irreversible nature of this shortening of the leg bones (Caskey and Norris, '40) also demonstrates the chick embryo's need for manganese for normal bone development.

Until recently, little information was available concerning the effects of manganese deficiency upon skeletal development in rats. Barnes et al. ('41) mention the observation of two "abnormal tibias" in 16 offspring of manganese-deficient rats. Shils and McCollum ('43) state briefly that "surviving young of markedly deficient females develop skeletal abnormalities. The gross effect is a shortening and bowing of the forelegs." Wachtel and associates ('43) found a lowered ash content in the bones of manganese-deficient rats. Recently Frost et al. ('59) have made a more extensive study of the skeletal deformities in manganese-deficient rats. Shortening and bowing of the forelegs was found to occur in 70 to 90% of the surviving young of deficient females at 30 and 60 days of age, respectively. Histologic study revealed retardation in osteogenesis and in skeletal maturation.

Although many of the experiments cited have referred to shortening of the limbs as a result of manganese deficiency, in no case has a systematic investigation of the growth of various components of the skeleton been made. It was therefore of interest to study the growth of the long bones in offspring of normal and manganese-deficient rats, with special emphasis on the early portion of the life span. The present paper describes the growth of the radius, ulna, tibia, and fibula as well as body length in normal and manganese-deficient young, from birth to 32 days of age. In addition, measurements made at adult ages are shown. The second paper of this series will present data on the growth of the skull.

METHODS

Weanling female rats of the Sprague-Dawley strain were purchased from com-

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mercial sources, and were maintained with a ration composed primarily of fresh, whole market milk, containing 13 μg of manganese per 100 ml (by spectrographic analysis). This was supplemented with minerals and vitamins as previously described (Hurley et al., '60).[2] For the control groups, manganese (as manganous sulfate) was added in amounts to provide 600 μg for each 100 ml of milk. At maturity, the animals were mated with normal males receiving a stock diet, and the resulting young were compared. Most of the young used in these studies came from second litters; in a few cases, first and third litters were used as well. The young were maintained with the diet received by their mothers (either manganese-deficient or manganese-supplemented) until the time they were sacrificed.

Measurements of crown-rump length,[3] and length of radius, ulna, tibia, and fibula were made with vernier calipers from specimens cleared and stained with alizarin red S, according to the method of Wright et al. ('58). In measuring the long bones, only diaphyseal length was used.[4] A total of 151 supplemented, and 162 deficient animals was studied. In addition, measurements of 41 adult rats were made from roentgenograms. The animals were anesthetized with ether for 40 to 60 seconds and the roentgenograms were taken on Eastman Industrial Type AA film.

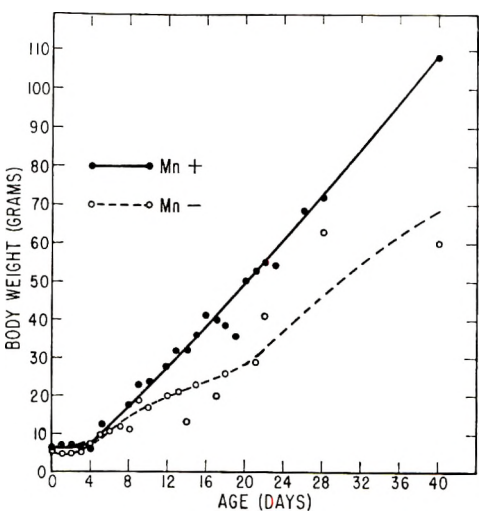

Fig. 1 Body weight in offspring of manganese-deficient and manganese-supplemented female rats. Each point represents the mean of from 5 to 30 animals.

RESULTS

Body weights of offspring as affected by manganese deficiency are shown in figure 1. No difference in body weight was apparent between the two groups at birth, but beginning about 6 days of age, body weight in the deficient animals was markedly lower than in the controls.

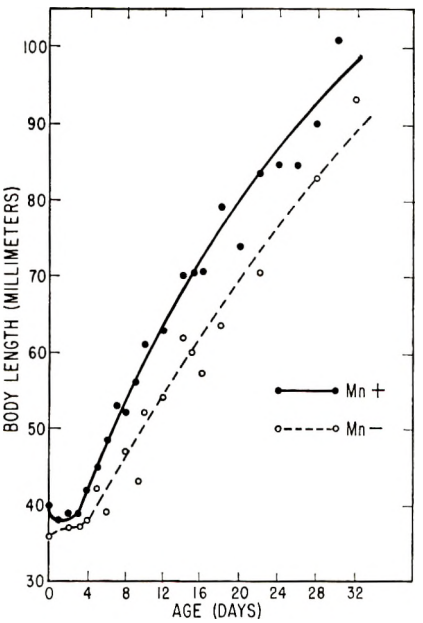

Fig. 2 Crown-rump length in offspring of manganese-deficient and manganese-supplemented female rats. Each point represents the mean of from three to 33 animals.

Body length in the two groups of animals is shown in figure 2. Manganese-deficient animals were consistently shorter than the controls, but the difference did not appear to be as great in the latter part of the period

[2] We are indebted to Merck Sharp and Dohme, Inc., Rahway, New Jersey, and to Hoffman-LaRoche, Inc., Nutley, New Jersey for supplies of vitamin B_{12} and ascorbic acid.

[3] Crown-rump length, rather than nose-anus length, was used in order to minimize the effect of an abnormality of skull length upon the body-length measurement.

[4] Diaphyseal length was used rather than total length, so that bone lengths could be compared irrespective of the presence or the absence of epiphyses.

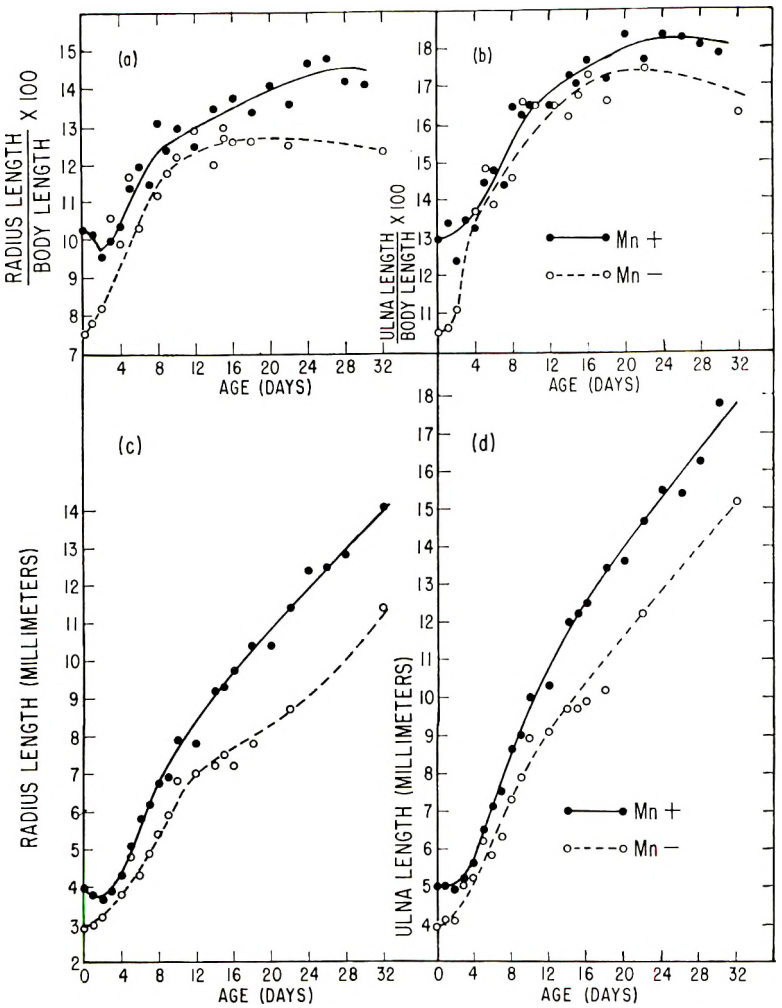

Fig. 3 Growth of radius and ulna in offspring of manganese-deficient and manganese-supplemented female rats. Each point represents the mean of from three to 33 animals. (a) Radius length relative to crown-rump length. (b) Ulna length relative to crown-rump length. (c) Absolute radius length. (d) Absolute ulna length.

studied as was true in the case of body weight.

Lengths of radius and ulna are shown in figure 3. Since body length as a whole was affected by the manganese deficiency, radius and ulna lengths are shown relative to body length (figs. 3a and 3b) as well as in absolute dimensions (figs. 3c and 3d). Growth of tibia and fibula is shown in figure 4, both absolute length (figs. 4c and 4d), and length relative to body length (figs. 4a and 4b).

In each of these bones, radius, ulna, tibia, and fibula, a striking difference between the normal and deficient offspring was apparent at birth. Both in terms of absolute length, and in terms of bone length relative to body length, the manganese-deficient newborn offspring showed a marked shortening of these bones. From 3 or 4 days of age to about 16 days of age, the growth of these bones relative to body length appeared to be the same in the two groups. After 16 days of age, the deficient animals again showed a marked decrease in the length of the long bones relative to body length.

Results of bone measurements from alizarin-stained specimens of a small number of older animals are summarized in

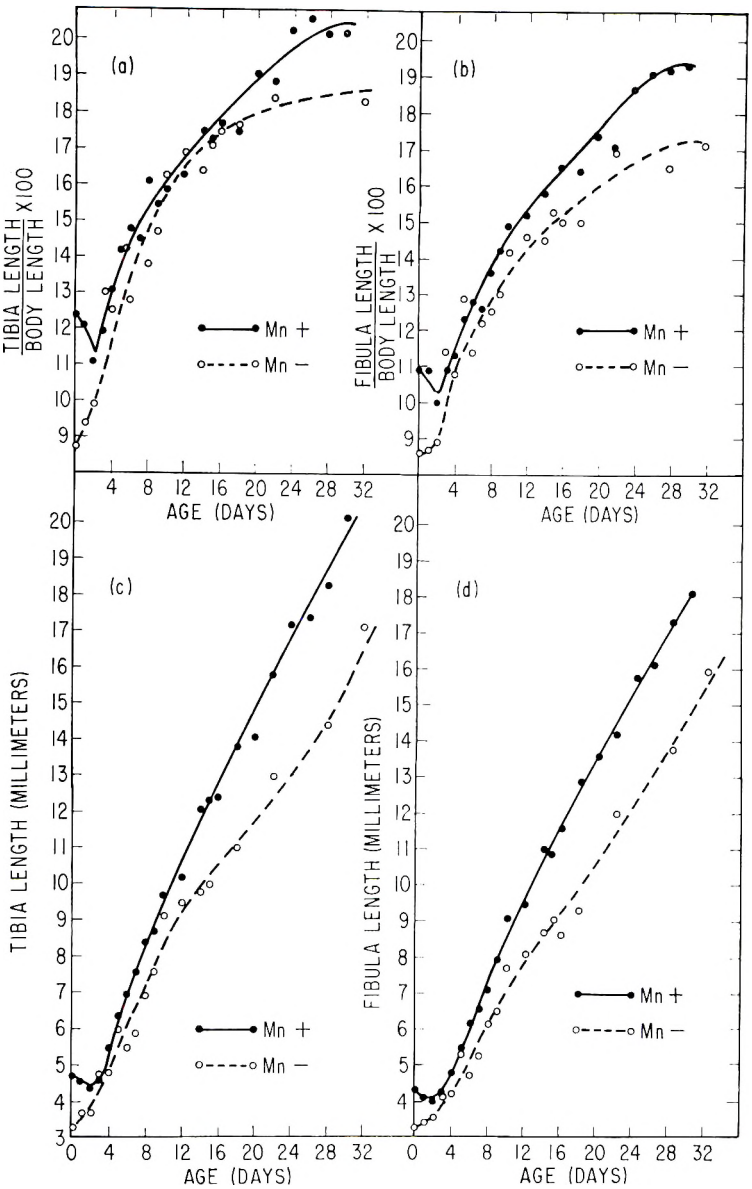

Fig. 4 Growth of tibia and fibula in offspring of manganese-deficient and manganese-supplemented female rats. Each point represents the mean of from three to 33 animals. (a) Tibia length relative to crown-rump length. (b) Fibula length relative to crown-rump length. (c) Absolute tibia length. (d) Absolute fibula length.

table 1. Bone measurements made from roentgenograms of adult rats are summarized in table 2. All of these animals were offspring of manganese-deficient or manganese-supplemented females, and were maintained with the respective diets until the ages stated. Data from both tables show that even at advanced ages, the long bones of the deficient animals were shorter relative to body length than those of the controls.

Examination of the alizarin-stained specimens revealed that the radii of the manganese-deficient rats were not only

TABLE 1

Length of long bones in manganese-deficient and normal adult rats (from alizarin-stained specimens)

Age	Sex	No. rats	Absolute lengths					Bone length/body length × 100			
			Body[1]	Radius	Ulna	Tibia	Fibula	Radius	Ulna	Tibia	Fibula
days			*mm*	*mm*	*mm*	*mm*	*mm*				
					Manganese-supplemented						
69	M	2	165.5	21.6	26.5	31.5	31.0	13.0	16.1	19.0	18.7
88	M	2	173.4	22.9	28.4	34.7	33.6	13.2	16.4	20.1	19.4
211	M	1	179.5	24.0	30.0	36.5	35.2	13.4	16.7	20.3	19.6
224	M	1	186.2	26.0	31.6	38.5	38.2	14.0	17.0	20.7	20.5
					Manganese-deficient						
81	F	1	160.7	19.0	24.5	28.5	28.6	11.8	15.2	17.7	17.8
200	M	3	161.3	19.3	25.2	30.1	29.5	12.0	15.7	18.7	18.2
200	F	1	151.0	19.9	25.7	31.2	30.2	13.2	17.0	20.7	20.0
222	F	1	160.0	20.0	25.6	29.9	29.5	12.5	16.0	18.7	18.4

[1] Crown-rump.

TABLE 2

Length of long bones in manganese-deficient and normal adult rats[1] (from roentgenograms)

Age	Sex	No. rats	Absolute lengths				Bone length/body length × 100		
			Body[2]	Radius	Ulna	Tibia	Radius	Ulna	Tibia
days			*mm*	*mm*	*mm*	*mm*			
					Manganese-supplemented				
87–94	M	10	158 ± 3	20.7 ± 0.0	26.4 ± 0.2	33.8 ± 0.3	13.2 ± 0.2	16.8 ± 0.2	21.5 ± 0.2
87–94	F	5	146 ± 2	19.3 ± 0.2	24.7 ± 0.4	31.4 ± 0.4	13.2 ± 0.1	17.0 ± 0.2	21.5 ± 0.2
224	M	4	178 ± 3	23.1 ± 0.2	29.7 ± 0.3	38.0 ± 0.5	12.9 ± 0.3	16.7 ± 0.2	21.3 ± 0.3
193	F	4	165 ± 4	21.0 ± 0.4	27.0 ± 0.3	33.5 ± 0.3	12.7 ± 0.4	16.4 ± 0.5	20.3 ± 0.4
Grand average, 23 rats							13.1 ± 0.1	16.7 ± 0.1	21.3 ± 0.2
					Manganese-deficient				
82–93	M	7	130 ± 3	16.3 ± 0.3	21.5 ± 0.4	25.5 ± 0.4	12.5 ± 0.3	16.5 ± 0.3	19.7 ± 0.4
82–93	F	3	144 ± 1	16.2 ± 0.6	22.4 ± 0.4	27.9 ± 0.9	11.2 ± 0.6	15.6 ± 0.4	19.4 ± 0.9
208–222	M	3	163 ± 4	17.5 ± 1.2	24.3 ± 1.1	31.1 ± 1.1	10.7 ± 0.5	14.5 ± 0.4	19.1 ± 0.3
208–236	F	5	147 ± 3	17.7 ± 0.5	23.3 ± 0.5	28.4 ± 0.7	12.1 ± 0.3	16.0 ± 0.2	19.5 ± 0.2
Grand average, 18 rats							12.3 ± 0.2[3]	16.0 ± 0.2[4]	19.5 ± 0.2[3]

[1] Means ± standard error.
[2] Crown-rump length. Measured in lateral view from the interparietal-parietal suture to the point of intersection of the rump with the spinal column.
[3] $P < 0.001$ as determined by Student's "t" test, grand average manganese-supplemented vs. deficient.
[4] $P < 0.01$ as determined by Student's "t" test, grand average manganese-supplemented vs. deficient.

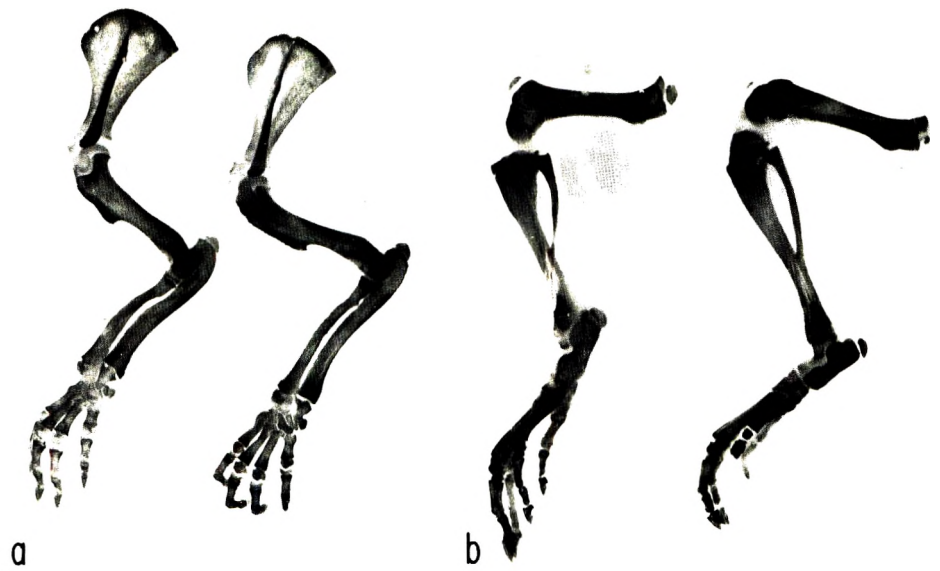

Fig. 5 Alizarin-stained preparations of fore and hind limbs of a manganese-deficient and a manganese-supplemented rat of the same body length, at 30 days of age. (a) Fore limbs. Deficient on left, control on right. (b) Hind limbs. Deficient on left, control on right. Note shortening and thickening of bones of deficient animals. Note also apparent absence of proximal tibial epiphysis (see footnote 5).

shorter, but were thicker than those of the controls. In most cases, a curvature of the radius was also observed. Comparison of deficient and normal animals of the same body length, however, indicated that the decreased length of the radius shown by the bone measurements was not an artifact resulting from the measurement of a curved bone. Likewise, tibias of deficient rats were greatly thickened and distorted in shape as compared with controls; but again, comparison of tibias from deficient and normal animals of the same body length showed that distortion or curvature of the bone could not account for the shortness of the tibia indicated by the measurements. Figure 5 shows examples of the appearance of these bones in animals at 30 days of age.[5]

DISCUSSION

The results presented here confirm and extend the previous reports (Barnes et al., '41; Shils and McCollum, '43; Frost et al., '59) that manganese deficiency in the maternal diet of rats results in a shortening of the limbs in the offspring. The results also show that the most pronounced shortening of the long bones is seen in newborn animals, and in those beyond 16 days of age.

In the present study a severe shortening of the radius, ulna, tibia, and fibula was apparent at birth. This finding of a congenital defect of bone development or growth is consistent with the observations of Lyons and Insko ('37) and Caskey et al. ('39) in the chick. The rat fetus, as well as the chick embryo, apparently requires adequate supplies of manganese if normal skeletal development is to occur. Data on skull measurements which also show disproportionate dimensions in the newborn deficient young (Hurley et al., '61) support this conclusion. The delayed ossification of the otic capsule observed earlier (Hurley et al., '60) may also be a manifestation of the same effect.

[5] In alizarin-stained specimens of month-old animals another, hitherto unreported, abnormality was observed, namely, a dysplasia of the proximal tibial epiphysis (seen in fig. 5). A detailed report of this finding will be presented in a subsequent publication.

Between the ages of 4 and 16 days, relative growth rates of the long bones were similar in normal and deficient young. No explanation for this finding is at hand, but it is possible that the pronounced difference between the two groups at birth, followed by the similarity at 4 days of age, represents the inclusion (in the deficient group) of newborn young which were severely affected, and which would have died during the first 4 days after birth. Survival of the manganese-deficient young is poor (Hurley et al., '58), and most of the deaths occur within the first 4 days. Although animals were selected randomly for sacrifice, the young available for sampling beyond the age of 4 days necessarily consisted only of those which had survived, and were presumably not as severely affected by the deficiency as those which had died.

Body weight also changed at about 4 days of age. From birth to 4 days, there was no difference in the body weight of the two groups, and no change in body weight occurred during this period. At 5 days of age, both groups began to show an increase in body weight, but a lower rate of increase became apparent in the deficient young at this time.

Likewise, no explanation is available for the difference in skeletal growth rates which occurred after 16 days of age between the two groups. Some physiological change must have occurred which did not permit normal proportional skeletal growth after this time, but the nature of this change is at present obscure.

Since body weight of the manganese-deficient offspring was lower than that of the controls, the question arises whether underfeeding might be a factor in the observed changes in bone growth. Jackson ('25), reviewing the effects of inanition upon growth, concluded that the skeleton is very resistant to depression of growth, and in fact, that "the most frequent change in the form of the body during dystrophic growth is an abnormal elongation, due to persistent skeletal growth. . ." More recent work by Berg ('60) also indicates that inanition is not responsible for the disproportionate growth observed here. Berg found that restriction of the food intake of rats to levels 33 and 46% below the ad libitum level, from weaning to 800 days of age, resulted in a 25 to 40% reduction in body weight. Tibia length and body length, however, were proportional (which was not true for the manganese-deficient rats described here), and linear plots gave points falling on the same straight line as the corresponding measurements of animals fed ad libitum. Thus, skeletal growth in animals subjected to caloric restriction (in contrast to those subjected to manganese deficiency) was in accord with Huxley's principle of heterogony ('32) that the ratio of relative growth rates between any two parts of the body, or a part of the body and the whole, remains constant.

The observation of shortening of the tibia described here is at variance with the results of Frost et al. ('59). These workers found no difference in tibia length relative to body length in manganese-deficient offspring as compared with controls, either at 30 or 60 days of age, although they did observe some bowing of this bone. In the data reported in the present paper, the shortening of the tibia is striking both in the newborn as well as in 30-day-old animals and adults.

Frost and co-workers also noted an ulnar deviation of the forepaw which could be seen at 5 days of age, but not in the newborn. In our studies, this ulnar deviation was often observed in newborn young of manganese-deficient females as a turning of the forepaws in the lateral direction. (Alizarin-stained preparations of the newborn, however, did not show this deformity.) The observation of this abnormality at birth, rather than at 5 days as in the case of Frost and his co-workers, indicates that the animals studied in the present investigation were in a more severe state of deficiency. (See also footnote 5.) This might account for the discrepancy with respect to tibia length.

The fact that the ulnar deviation could be observed in the intact animal at birth, but not in alizarin-stained preparations, suggests that the primary defect may be in the cartilage model. Frost et al. also suggest the possibility of abnormal cartilage formation on the basis of histologic study of the epiphyseal cartilage perforations which they observed in the tibia. Wolbach and Hegsted ('53), in an extensive study of histologic changes in perosis caused by manganese and choline

deficiencies in the chick, have concluded that manganese is not required for osteogenesis *per se*, but is essential for epiphyseal cartilage cell metabolism.

SUMMARY

Growth of long bones was studied in offspring of normal and manganese-deficient rats by measuring the length of radius, ulna, tibia, and fibula, as well as body length, in alizarin-stained preparations of animals from birth to 32 days of age and in adult animals from roentgenograms. Body lengths of deficient offspring were consistently shorter than normal. Each of the long bones measured was strikingly shorter than normal in the deficient offspring at birth, both in terms of absolute length, and in terms of bone length relative to body length. From three or 4 days of age to about 16 days of age, the growth of these bones relative to body length appeared to be the same in the two groups. After 16 days of age, the deficient animals again showed a marked decrease in the length of the long bones relative to body length. The radii and tibias of deficient rats were greatly thickened and distorted in shape as compared with the controls. In most cases, a curvature of the radius was seen.

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Disproportionate Growth in Offspring of Manganese-Deficient Rats

II. SKULL, BRAIN AND CEREBROSPINAL FLUID PRESSURE[1]

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Although there have been numerous examples of the importance of manganese for normal development of the long bones (Underwood, '56; Cotzias, '58; Hurley et al., '61), references to the influence of manganese on the growth of the skull are only fragmentary. Lyons and Insko ('37) observed a globular contour of the head in chick embryos from manganese-deficient hens. Caskey and Norris ('40) also mentioned the observation of abnormally shaped heads in hens grown from manganese-deficient eggs.

In paper I of this series (Hurley et al., '61), concerning the effect of manganese deficiency upon growth in body weight, body length, and 4 long bones, severe shortening of the long bones was described. The observation of abnormally shaped skulls in these offspring of manganese-deficient rats led to a study of this portion of the skeleton as well.

One of the striking effects of a maternal dietary deficiency of manganese is a congenital ataxia in the young (Hurley et al., '58); the etiology of this ataxia is poorly understood (Hurley et al., '60). Although we have not observed hydrocephalus in young from manganese-deficient mothers, it was nevertheless of interest to study growth of the skull in relation to growth of the brain. In vitamin A-deficient animals, ataxia and paralysis appeared to result from a disproportion between growth of the skull and of the brain (Wolbach and Bessey, '41). An increase in the cerebrospinal fluid pressure was also observed (Moore and Sykes, '40; Mellanby, '41). Millen and Woollam ('56), however, found some instances of young born to vitamin A-deficient rabbits that showed an increase in cerebrospinal fluid pressure before the appearance of hydrocephalus. It therefore seemed important in the present studies to determine whether increased intracranial pressure (as measured by cerebrospinal fluid pressure) existed in manganese-deficient young.

The present paper describes the growth of the skull in offspring of normal and manganese-deficient rats from birth to 32 days of age, and in adults. Growth of the brain is also presented. Cerebrospinal fluid pressure values in animals from three weeks to 6 months of age are shown.

METHODS

The animals used in this study were the same as those for which long bone measurements were reported in the first paper of this series (Hurley et al., '61).[2]

Measurements of skull dimensions from birth to 32 days of age were made with vernier calipers from specimens cleared and stained with alizarin red S, according to the method of Wright et al. ('58). One-hundred-fourteen deficient animals, and 128 control animals were measured. Skull length was considered the distance from the tip of the nasal bone to the foramen magnum; skull width was measured at the widest portion of the skull and included the zygomatic arches; skull height was measured from the point of highest curvature of the skull in a line perpendicular to the base of the mandible. In addition,

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measurements of individual skull bones of 33 newborn young were made in the midline. Skull dimensions were also measured in 33 adult rats from roentgenograms. The animals were anesthetized lightly with ether and the roentgenograms were taken on Eastman Industrial Type AA film.

A different group of 165 animals was decapitated at the time of sacrifice, and the brains were removed and weighed. Dry weight was determined in 77 brains after drying at 60°C to constant weight. In another group of animals, cerebrospinal fluid pressure was measured with the use of a bubble manometer, by puncture of the cisterna magna, according to the method of Jeffers and Griffith ('49).

RESULTS

Measurements of skull length from birth to 32 days are shown in figure 1. Some depression of lengthwise growth of the skull was apparent throughout this period in deficient animals. Skull width and skull height are shown in figure 2. Since skull length was affected by the manganese deficiency, skull width and height are shown in relation to skull length (figs. 2a and 2b), as well as in absolute dimensions (figs. 2c and 2d).

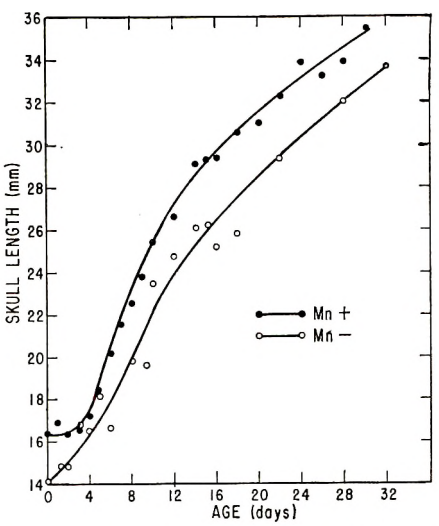

Fig. 1 Skull length in offspring of manganese-deficient and manganese-supplemented female rats. Each point represents the mean of from three to 30 animals (in most cases, 5 to 7).

The difference between normal and manganese-deficient offspring with respect to skull proportions is striking. Both skull width and skull height were only slightly smaller in the deficient young on an absolute basis. When these dimensions are considered in proportion to the length of the skull, however, both the width and height were greater in the deficient animals. In both cases, the difference was very large at birth. The skulls of the newborn offspring of deficient females were markedly wider and higher in proportion to their length than those of the controls. This difference was apparent grossly; deficient newborns had visibly domed skulls. Deformed alizarin-stained skulls of newborns have been illustrated previously (Hurley et al., '60).

Midline measurements of the skull of newborn animals were made to analyze further this doming of the newborn skull. Figure 3a shows the dimensions which were measured. "A" represents the distance, measured with calipers, from the base of the interparietal bone to the highest point on the skull. "B" is the distance from the highest point of the skull to the tip of the nasal bone, and "C" is the length of the skull from the base of the interparietal bone to the tip of the nose. In addition, the length (as measured in midline with calipers) of the interparietal and parietal, the frontal, and the nasal bones is shown. The results of these measurements are summarized in table 1. Although "A" was somewhat (5%) longer in the deficient animals, both "B" and "C" were reduced (by 14 and 11%, respectively). Length of the interparietal and parietal bones was the same in both groups, but the frontal and nasal bones were 6 and 11%, respectively, shorter in the deficient animals. The ratio (A + B)/C is a numerical representation of the difference in skull proportions between the two groups. Figure 3b represents the mean figures for the two groups, drawn to scale, and provides a diagrammatic representation of the difference in appearance between the normal and deficient animals.

Skull dimensions of adult rats, as measured from roentgenograms taken in standardized planes (Asling, '51), are summarized in table 2. Skull width in relation to

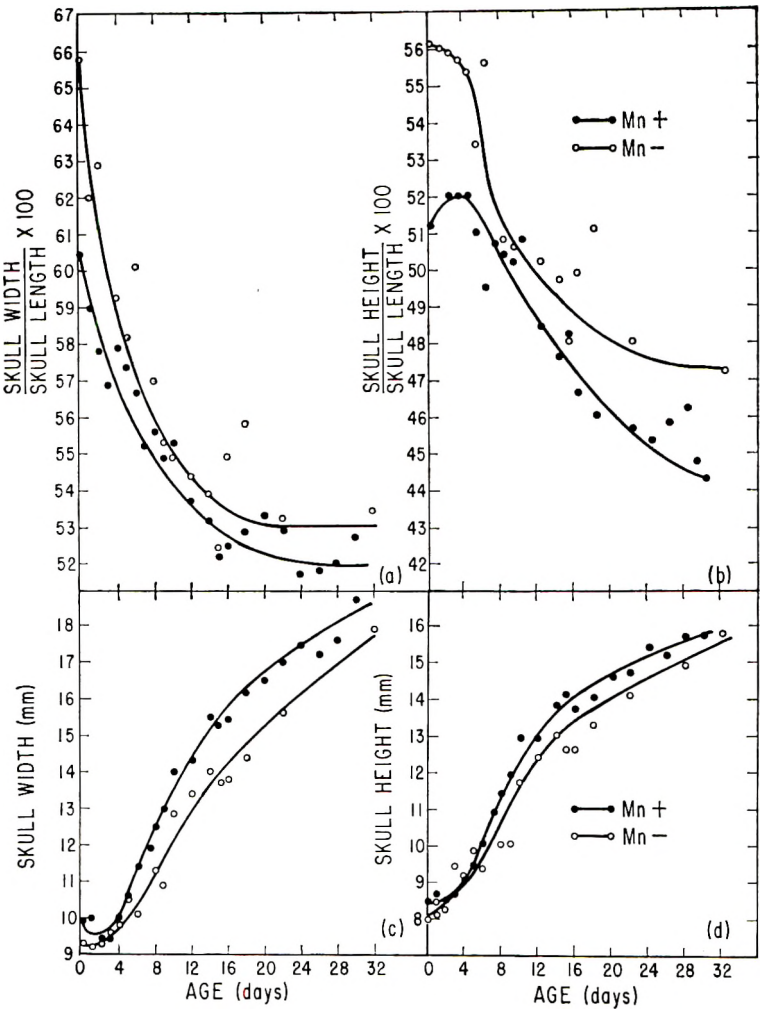

Fig. 2 Skull width and height in offspring of manganese-deficient and manganese-supplemented female rats. Each point represents the mean of from three to 30 animals (in most cases, 5 to 7). (a) Skull width relative to skull length. (b) Skull height relative to skull length. (c) Absolute skull width. (d) Absolute skull height.

skull length was larger in the deficient animals, but the difference as compared with the controls was not statistically significant. The ratio of skull height to skull length, on the other hand, showed a statistically significant increase in the deficient rats as compared with the supplemented animals.

Observations on growth of the brain are shown in figure 4. In absolute terms (fig. 4a), brain weight of the two groups was the same at birth, and did not show a difference until after 4 days of age. After this time, however, growth of the brain was depressed in the deficient young. Since body weight as a whole was affected by the manganese deficiency (Hurley et al., '61), brain weight is also expressed in relation to body weight (fig. 4b). On this basis, there was a pronounced difference between the two groups. After 4 days of age, the brains of the deficient young were larger in relation to body weight than those of the controls.

Dry weights of the brain are shown in table 3. From birth to 40 days of age, there was no significant difference in dry

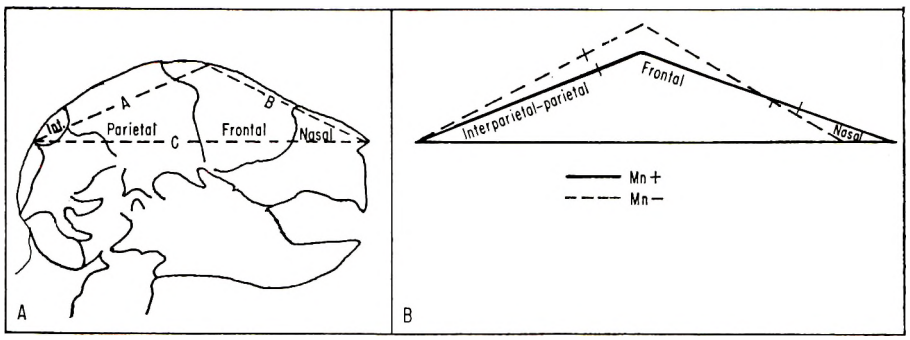

Fig. 3 (A) Diagram of rat skull showing dimensions measured in midline in newborn rats. "A" is the distance from the base of the interparietal bone to the point of highest curvature of the skull. "B" is the distance from the point of highest curvature of the skull to the tip of the nasal bone. "C" is the distance from the base of the interparietal bone to the tip of the nasal bone. (B) A diagrammatic representation of the results of skull measurements made in midline in newborn young. Each triangle is drawn to scale using the mean values for the respective group. The solid lines represent the skull measurements of normal (manganese-supplemented) animals; the broken lines represent the skull measurements of manganese-deficient rats. The short lines perpendicular to those representing dimensions "A" and "B" indicate the lengths of the cranial bones: interparietal + parietal, frontal, and nasal.

TABLE 1

Skull dimensions in newborn young in midline[1]

Measurement[2]	Mn-supplemented	Mn-deficient	Difference
	mm	*mm*	*%*
No. of rats	18	15	
A	8.2 ± 0.14	8.6 ± 0.12	5[3]
B	9.1 ± 0.13	7.8 ± 0.12	14[4]
C	16.2 ± 0.13	14.4 ± 0.17	11[4]
Interparietal + parietal	6.6 ± 0.11	6.4 ± 0.11	3
Frontal	6.9 ± 0.05	6.5 ± 0.09	6[4]
Nasal	3.7 ± 0.04	3.3 ± 0.07	11[4]
$\frac{A+B}{C}$	1.07 ± 0.007	1.14 ± 0.006	6[3]

[1] For dimensions measured, see figure 3.
[2] Mean ± standard error.
[3] $P < 0.01$, as determined by Student's "t" test.
[4] $P < 0.001$.

weight of the brain between normal and manganese-deficient young.

Cerebrospinal fluid pressure in normal and deficient young from three weeks to 6 months of age is shown in table 4. The range of values obtained in both groups was large, but no significant differences were seen at any of the ages examined.

DISCUSSION

The results show that a profound alteration in skull proportions takes place when the developing rat is subjected to an inadequate supply of manganese. Skulls of manganese-deficient young were shorter, wider, and higher than those of normal controls, both at birth and thereafter. The shortening of the skull in the newborn, however, was not due to a proportional depression of growth in the cranial bones: length of the interparietal and parietal bones in newborn deficient young was similar to that in controls, and length of the frontal bone was less reduced than that of the nasal or than the length of the skull as a whole. Rather, the shortening appeared to be due largely to an inhibition of growth in the basal portion of the skull. This shortening of the skull, concomitant with dissimilar growth rates in the cranial bones, resulted in doming of the frontal portion of the skull.

TABLE 2

Skull measurements of manganese-deficient and normal adult rats[1] (from roentgenograms)

Age	Sex	No. rats	Absolute dimensions			Dimension / skull length × 100	
			Width	Length	Height	Width	Height
days			*mm*	*mm*	*mm*		
			Manganese-supplemented				
87–94	M	8–10	23.2 ± 0.22	45.5 ± 0.37	19.9 ± 0.46	51.2 ± 0.40	43.7 ± 1.0
87–94	F	2–4	22.4 ± 0.17	43.2 ± 0.64	20.1 ± 0.66	52.0 ± 0.53	45.7 ± 0.00
224	M	4	25.8 ± 0.33	48.3 ± 0.24	22.4 ± 0.12	53.5 ± 0.65	46.5 ± 0.29
193	F	3–4	24.0 ± 0.27	46.1 ± 0.72	20.3 ± 0.33	52.2 ± 0.00	44.1 ± 0.75
Grand average, 19 rats						52.0 ± 0.31	44.6 ± 0.36[2]
			Manganese-deficient				
82–93	M	7	21.6 ± 0.29	41.5 ± 0.42	18.9 ± 0.19	52.1 ± 0.61	45.5 ± 0.40
82–93	F	3	21.1 ± 0.21	39.7 ± 0.73	18.7 ± 0.09	53.2 ± 0.11	47.3 ± 0.97
208	M	2	23.1 ± 0.20	44.9 ± 0.30	21.3 ± 0.05	51.5 ± 0.71	47.3 ± 0.50
208–236	F	4	23.7 ± 0.30	44.3 ± 0.52	19.9 ± 0.29	53.6 ± 0.47	45.0 ± 0.91
Grand average, 16 rats						52.6 ± 0.38	45.9 ± 0.39[2]

[1] Mean ± standard error.
[2] $P < 0.05$, as determined by Student's "t" test.

Asling and his associates ('52) have studied the changes in skull proportions which take place after hypophysectomy in rats. In these animals there was also a marked failure of lengthwise growth, whereas skull width and height were almost normal. As a result, the disproportions were in the same direction as those observed in the present study. Histologic examination revealed that the chief factor in the growth arrest was the cessation of endochondral osteogenesis in the base of the skull. (Administration of growth hormone induced nearly normal growth.) Although direct comparisons with the present study are difficult because of the difference in ages measured (in Asling's study, measurements were not begun until the animals were 30 days old), it is interesting to speculate that growth hormone or its target, endochondral ostoegenesis, plays some role in the abnormal skeletal development of animals lacking manganese. This suggestion is consistent with the conclusion of Wolbach and Hegsted ('53) that manganese is essential for epiphyseal cell metabolism.

Manganese deficiency also had a marked effect on growth of the brain. In absolute terms, brain weight was depressed, but relative to body weight, the brains of deficient animals were larger than those of controls. Determination of dry weights showed that this increase in mass was not the result of edema, but was due to maintenance of brain growth in the face of diminished body growth. This finding, however, is not necessarily indicative of a specific effect of manganese deficiency on brain growth. In general, growth of the brain is little affected by conditions which may produce alterations in the growth of other organs (Jackson, '25).

Cerebrospinal fluid pressure measurements were not significantly different in the two groups. It is possible, however, that a small difference would be masked by the wide range of values which was obtained even in normal animals, and which seems to be inherent in the method (Davson, '56). On the other hand, measurements of cerebrospinal fluid pressure in vitamin A-deficient animals (Millen and Dickson, '57) have revealed an obvious elevation of pressure, despite an equally

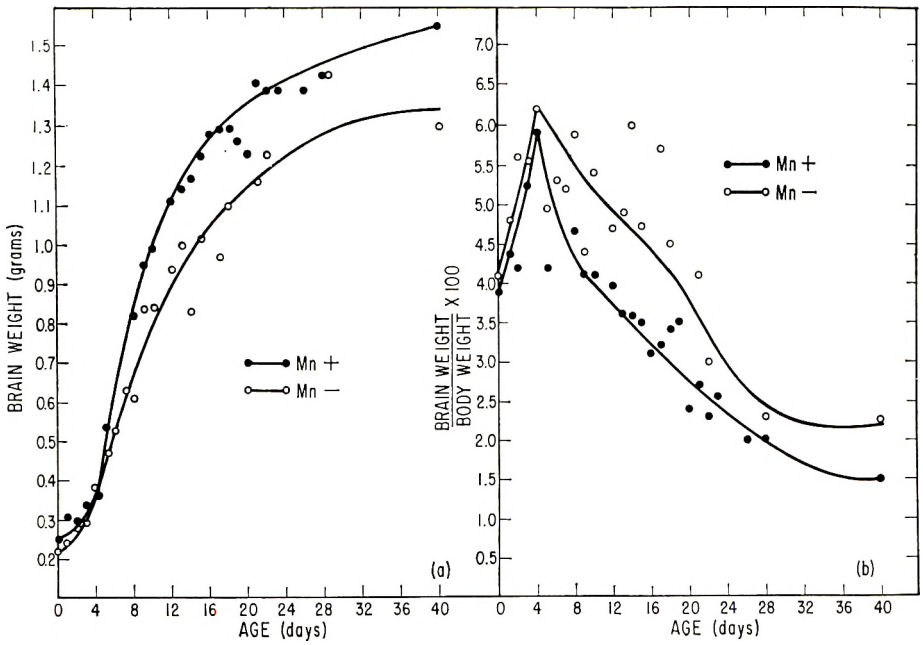

Fig. 4 Brain weight in offspring of manganese-deficient and manganese-supplemented female rats. Each point represents the mean of from three to 15 animals. (a) Absolute brain weight. (b) Brain weight relative to body weight.

TABLE 3

Dry weights of brains in offspring of manganese-deficient and normal rats

Age	Dry weights			
	Normal		Deficient	
days	*% of fresh weight*		*% of fresh weight*	
Newborn	12.73[1]	(5)[2]	12.79[1]	(7)[2]
1	12.50	(5)	13.00	(5)
2	12.31	(5)	13.39	(4)
4	12.64	(5)	13.76	(5)
7	12.36	(5)	12.54	(1)
14	15.10	(6)	15.78	(5)
40	20.64	(9)	20.65	(10)

[1] Mean.
[2] Number of animals.

large variability. In any case, the present results do not suggest that intracranial pressure is a factor in the ataxia of manganese-deficient offspring.

SUMMARY

Growth of the skull was studied in offspring of normal and manganese-deficient rats from birth to 32 days of age by measuring skull length, width, and height in alizarin-stained preparations. The deficient animals showed some depression of lengthwise growth of the skull throughout this period. Skull width and skull height were also slightly decreased in the defi-

TABLE 4

Cerebrospinal fluid pressure in offspring of manganese-deficient and normal rats

Age	Cerebrospinal fluid pressure			
	Normal		Deficient	
	mm H_2O	*No. rats*	*mm H_2O*	*No. rats*
3 weeks	77[1] (59– 94)[2]	8	—	—
1 month	75 (56–107)	24	80[1] (60–103)[2]	10
2 months	91 (73–135)	31	—	—
3–4 months	84 (70–100)	12	77 (70– 86)	9
5–6 months	87 (71–107)	9	91 (79–113)	8

[1] Mean.
[2] Range.

cient animals on an absolute basis. In relation to their length, however, the skulls of deficient young were wider and higher than those of controls. This difference was very marked at birth. Midline measurements of the skulls of newborn young indicated that the doming of the frontal portion of the skull in deficient offspring resulted from an inhibition of growth in the basal portion of the skull concomitant with dissimilar rates of growth in the cranial bones. Skull dimensions of manganese-deficient adult rats, measured from roentgenograms, also showed an increased ratio of skull height to skull length, although the ratio of width to length was not significantly increased.

Brain weights were smaller in the deficient animals on an absolute basis, but in relation to body weight the brains of deficient young were larger than those of controls. Moisture determinations of the brain tissue showed that the increase was not caused by edema.

Cerebrospinal fluid pressure was measured in normal and deficient young from three weeks to 6 months of age. No significant differences between the two groups were seen in this respect.

ACKNOWLEDGMENT

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Effect of Vitamin B_6 on the Growth of Rats Fed Diets Limiting in an Essential Amino Acid and on the Utilization of Isomers of Tryptophan, Methionine and Valine[1,2]

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Various reports have clearly demonstrated that several of the D-amino acids or corresponding keto- or hydroxy-acids, in the presence of vitamin B_6, can replace the corresponding L-amino acid in the nutrition of certain micro-organisms (Williams, '48; Holden and Snell, '49; Camien and Dunn, '50; Holden et al., '51; Snell, '52, '53; Sebrell and Harris, '54). Although several D-amino acids are active for the growth of the rat (Williams, '48; Wretlind and Rose, '50; Berg, '53; Snell, '53; Phillips and Berg, '54; Wretlind, '52a, b, '56), little information is available as to the influence of vitamin B_6 on this activity. It is considered, however, that vitamin B_6 is generally required for enzymatic reactions involving the non-oxidative degradation and interconversion of amino acids (Snell, '52, '53; Sebrell and Harris, '54). The present studies were initiated in an attempt to determine whether the level of vitamin B_6 in the diet would influence the growth of rats fed diets limiting in an amino acid and, as well, the growth-promoting activity of several of the essential L-amino acids and their corresponding D- isomers.

EXPERIMENTAL

Male weanling rats of the Sprague-Dawley strain were housed individually in wire-bottom cages in an air-conditioned room. Food and water were given ad libitum and the animals were weighed twice a week. Rats were depleted of pyridoxine by feeding a pyridoxine-free 20% casein diet for 7 or 10 days before being placed on experiment. Composition of the basal diets used is given in table 1. In diets 3 and 6, the protein was replaced by an amino acid mixture. The composition of the amino acid mixture was the result of other studies in this laboratory (Sauberlich, '61). Levels of supplementation with pyridoxine hydrochloride and amino acids are indicated in the tables. Blood samples were obtained by heart puncture from certain groups of animals at the termination of the experiment. Protein-free filtrates were prepared (Hier and Bergeim, '45) and analyzed for tryptophan, histidine, and glycine content by microbiological procedures with the aid of *Pediococcus pentosaciens*.[4] The purity of the amino acid supplements fed was also checked by microbiological procedures.

RESULTS

The effects of dietary supplements of pyridoxine on the growth of rats fed controlled amounts of D- or L-tryptophan are presented in tables 2 and 3. In table 2, the results were obtained with rats fed basal diet 2 containing oxidized casein (free of tryptophan) as the source of pro-

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[3] Present address: U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver 30, Colorado.

[4] Sauberlich, unpublished data.

TABLE 1

Composition of the experimental diets (gm/kg of diet)

	Diet no.[1]					
	1	2	3	4	5	6
Extracted casein[2]	100	—	—	100	—	—
Oxidized casein[3]	—	100	—	—	—	—
Alpha soy protein[4]	—	—	—	—	120	—
Amino acid mix	—	—	249.5[5]	—	—	116[6]
Salts[7]	50	50	50	50	50	50
Corn oil	40	40	—	40	40	—
Lard	—	—	150	—	—	150
Cod liver oil	10	10	10	10	10	10
Cellulose[8]	—	—	20	—	—	20
Sucrose	794	790	517.5	797	777	651
Choline	2	2	2	2	2	2
L-Cystine	3	3	—	—	—	—
L-Methionine	—	4	—	—	—	—
Inositol	1	1	1	1	1	1

[1] All diets were supplemented with the following vitamins (mg/kg of diet): Ca pantothenate, 30; α-tocopheryl acetate, 100; niacin, 25; riboflavin, 6; thiamine, 6; 2-methyl-1,4-naphthoquinone, 5; biotin, 0.5; folacin, 2; and vitamin B_{12}, 0.05. Pyridoxine was added to the above diets at levels indicated in the tables or text. Additional supplements as indicated in tables were added at the expense of sucrose.

[2] Schaefer and Knowles, '51.

[3] Hove et al., '49.

[4] Drackett C-1 Assay Alpha Soy Protein obtained from the Drackett Company, Cincinnati. The protein as received was washed thoroughly with water, dried, and extracted for 24 hours with hot methanol in a continuous extractor.

[5] The amino acid mixture was composed of the following: (in gm) DL-alanine, 6.0; L-arginine·HCl, 8.0; L-aspartic acid, 6.0; L-asparagine H_2O, 6.0; L-cystine, 3.0; L-glutamic acid, 40.0; glycine, 4.0; L-histidine·HCl, 10.0; L-leucine, 20; DL-isoleucine, 30.0; L-lysine·HCl, 18.5; DL-methionine, 8.0; DL-phenylalanine, 13.0; L-proline, 5.0; DL-serine, 5.0; DL-threonine, 15.0; L-tyrosine, 8.0; DL-valine, 28.0; and $NaHCO_3$, 16.0.

[6] The amino acid mixture was composed of the following: (in gm) DL-alanine, 3.0; L-arginine·HCl, 4.0; L-aspartic acid, 3.0; L-asparagine H_2O, 3.0; L-cystine, 3.0; L-glutamic acid, 20.0; glycine, 2.0; L-histidine·HCl, 5.0; L-leucine, 10.0; DL-isoleucine, 15.0; L-lysine·HCl, 9.0; DL-methionine, 4.0; DL-phenylalanine, 6.5; L-proline, 2.5; DL-serine, 2.5; DL-threonine, 9.0; DL-tryptophan, 2.5; L-tyrosine, 4.0; and $NaHCO_3$, 8.0.

[7] Salmon, '47.

[8] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

tein and with controlled amounts of tryptophan and pyridoxine. The diets were supplemented with L- or D-tryptophan at a level of 0.1 or 0.2% of the diet and with pyridoxine hydrochloride at a level of either 0.5, 1.0, or 6.0 mg per kg of diet. From these studies it was observed that the growth of the animals could be improved by supplements to the diet of *either pyridoxine or tryptophan or both.* This was true for both L-tryptophan and D-tryptophan. At the 0.1% level of the diet, however, D-tryptophan was inferior to L-tryptophan regardless of the level of pyridoxine fed (table 2). Moreover, D-tryptophan at the 0.1% level of the diet appeared to require the presence of greater amounts of pyridoxine in the diet for activity than did a corresponding level of L-tryptophan. The degree of pyridoxine depletion in the animals is indicated by the growth of the control animals fed graded levels of the vitamin.

Similar studies were carried out with diets containing a purified amino acid mixture (free of tryptophan) in place of the oxidized casein (diet 3). This diet permitted improved growth of the animals and a better control of the tryptophan and pyridoxine content. The results of these studies are presented in table 3 and are similar to those obtained with the oxidized casein diets (table 2). Growth of the animals was again improved by supplements of either pyridoxine or D-tryptophan. It was also observed, however, that D-tryptophan required for activity the presence of more pyridoxine in the diet than was re-

TABLE 2

Effect of vitamin B_6 on the utilization of tryptophan by weanling rats[1] *(oxidized casein basal diet)*

Group no.	Basal diet no.	Diet	Vitamin B_6 supplement[2]	Av. daily food intake	Av. weight gain in 4 weeks	Av. gain/gm of food intake
			mg/kg diet	*gm*	*gm*	*gm*
		Experiment 1				
1	2	Basal[3] + 0.1% L-tryptophan	0.5	—	34	—
2	2	Basal + 0.1% L-tryptophan	1.0	—	47	—
3	2	Basal + 0.1% L-tryptophan	6.0	—	60	—
4	2	Basal + 0.2% L-tryptophan	0.5	—	44	—
5	2	Basal + 0.2% L-tryptophan	1.0	—	57	—
6	2	Basal + 0.2% L-tryptophan	6.0	—	63	—
7	2	Basal + 0.1% D-tryptophan	0.5	—	14	—
8	2	Basal + 0.1% D-tryptophan	1.0	—	18	—
9	2	Basal + 0.1% D-tryptophan	6.0	—	27	—
10	2	Basal + 0.2% D-tryptophan	0.5	—	45	—
11	2	Basal + 0.2% D-tryptophan	1.0	—	62	—
12	2	Basal + 0.2% D-tryptophan	6.0	—	66	—
13	1	Regular casein, 10%	0.5	—	57	—
14	1	Regular casein, 10%	1.0	—	88	—
15	1	Regular casein, 10%	6.0	—	106	—
		Experiment 2[4]				
16	2	Basal[3] + 0.1% L-tryptophan	0.5	7.1	21	0.14
17	2	Basal + 0.1% L-tryptophan	1.0	8.8	31	0.17
18	2	Basal + 0.1% L-tryptophan	6.0	9.4	47	0.24
19	2	Basal + 0.2% L-tryptophan	0.5	8.6	31	0.17
20	2	Basal + 0.1% D-tryptophan	0.5	7.9	15	0.09
21	2	Basal + 0.1% D-tryptophan	1.0	6.8	17	0.12
22	2	Basal + 0.1% D-tryptophan	6.0	8.4	32	0.18
23	2	Basal + 0.2% D-tryptophan	0.5	7.8	30	0.18
24	1	Regular casein, 10%	0.5	9.8	52	0.25
25	1	Regular casein, 10%	1.0	12.1	75	0.30
26	1	Regular casein, 10%	6.0	13.1	87	0.35

[1] Four rats used per group; rats depleted for 7 days with a pyridoxine-free 20% casein diet before starting on experiment; average initial weight after depletion was in experiment 1, 56 gm; in experiment 2, 52 gm.

[2] Pyridoxine hydrochloride.

[3] Basal diet contained 10% of oxidized casein free of tryptophan, supplemented with cystine and methionine.

[4] Weight gains and food intake values are for a three-week period.

quired when L-tryptophan was used. Thus, at a level of 0.5 mg of pyridoxine hydrochloride per kg of diet, 0.1% of L-tryptophan permitted a gain in weight of 21 gm in three weeks, whereas a comparable level of D-tryptophan permitted no gain. In the presence of 6 mg of pyridoxine per kg of diet, the 0.1% level of D-tryptophan permitted growth comparable to that of 0.1% level of L-tryptophan in the presence of 1.0 mg of pyridoxine per kg of diet.

In the presence of optimum amounts of pyridoxine, 0.1% of D-tryptophan in the diet permitted about 50% of the growth observed with L-tryptophan fed at a comparable level (32 gm vs. 68 gm gain in three weeks). With an adequate level of pyridoxine in the amino acid mixture diet, a level of 0.15% of L-tryptophan permitted an average weight gain per rat of 35 gm per week. As the level of pyridoxine or tryptophan in the diet was increased, the efficiency of gain (gain per gram of food intake) was improved.

Analyses for several plasma free-amino acids were made on rats fed controlled

TABLE 3

Effect of vitamin B_6 on the utilization of tryptophan by weanling rats[1] *(amino acid mix basal diet)*

Group no.	Basal diet no.	Diet	Vitamin B_6 supplement[2]	Av. daily food intake	Av. weight gain in 3 weeks[3]	Av. gain/gm of food intake
			mg/kg diet	*gm*	*gm*	*gm*
1	3	Basal[4]+0.1% L-tryptophan	0.5	5.8	21±2	0.17
2	3	Basal +0.1% L-tryptophan	1.0	6.9	36±2	0.25
3	3	Basal +0.1% L-tryptophan	6.0	9.2	68±3	0.36
4	3	Basal +0.15% L-tryptophan	6.0	10.7	106±4	0.48
5	3	Basal +0.20% L-tryptophan	6.0	10.7	107±3	0.48
6	3	Basal +0.1% D-tryptophan	0.5	5.6	1±1	0.003
7	3	Basal +0.1% D-tryptophan	1.0	6.4	4±1	0.03
8	3	Basal +0.1% D-tryptophan	6.0	8.5	32±2	0.18
9	3	Basal +0.2% D-tryptophan	0.5	8.4	48±3	0.27

[1] Four rats used per group; rats depleted for 10 days with a pyridoxine-free 20% casein diet before starting on experiment. Average initial weight after depletion was 71 gm.
[2] Pyridoxine hydrochloride.
[3] Average ± standard error of the mean.
[4] The basal diet contained an amino acid mix in place of intact protein and was devoid of tryptophan.

amounts of tryptophan and pyridoxine. Observations indicated that as the level of tryptophan (D or L) or pyridoxine hydrochloride was increased, the concentration of free glycine in the plasma decreased, and that of histidine remained nearly unchanged. Thus, glycine decreased from a level of 47 ± 6.5 μg per ml of plasma in rats fed the 0.1% L-tryptophan diet containing 0.5 mg of pyridoxine per kg to a level of 16.8 ± 1.0 μg per ml of plasma when receiving the 6.0 mg level of pyridoxine. Plasma free-tryptophan levels decreased, when the amount of pyridoxine fed was increased in the presence of only 0.1% of L-tryptophan in the diet (8.6 ± 2.3 μg of tryptophan per ml of plasma to 4.6 ± 0.2 μg per ml). A similar decrease in plasma tryptophan was noted when D-tryptophan was used. At this level of the amino acid, however, L-tryptophan supported somewhat higher plasma levels of tryptophan than did D-tryptophan. The higher level of dietary tryptophan (0.2% of the diet) increased the plasma tryptophan level, which was enhanced slightly by the higher level of pyridoxine. The microbiological assay procedure used measured only L-tryptophan.

In table 4 are presented data from a similar study on the influence of pyridoxine on the activity of L- and D-methionine for the growth of weanling rats. In experiment 1, the methionine-low diet was produced with the use of casein, whereas in experiment 2 alpha soy protein was was used. The alpha soy protein was thoroughly washed with water and extracted extensively with hot methanol prior to use in the present investigations. In contrast to the findings of DeBey et al. ('58), methionine supplements produced marked responses in the growth of the animals fed the 12% soy protein diet.

From the results presented, it may be observed that supplements of *either pyridoxine or methionine or both improved growth of the animals*, an effect similar to that observed with tryptophan. Thus, for example, in experiment 2 with the soy protein diet (table 4), the average weight gain per rat in a 4-week period was increased from 32 to 55 gm when the level of pyridoxine in the diet was increased from 0.5 to 6.0 mg per kg of diet; a gain of 72 gm was noted when a supplement of 0.1% of L-methionine was added to the low-pyridoxine diet. In addition, at the low level of pyridoxine (0.5 mg per kg of diet), 0.1% of L-methionine permitted more growth than a comparable level of D-methionine (experiment 2; group 12 vs. 18). When the level of pyridoxine was increased to 1 or 6 mg per kg of diet, the two isomers of methionine were then of equal activity. At a level of 0.2% of methionine

TABLE 4

Effect of vitamin B_6 on the utilization of methionine by weanling rats

Group no.	Basal diet no.	Diet	Vitamin B_6 supplement[1]	Av. daily food intake	Av. weight gain in 4 weeks[2]	Av. gain/gm of food intake
			mg/kg diet	*gm*	*gm*	*gm*
		Experiment 1[3]				
1	4	Basal (10% casein)	0.5	8.2	52	0.23
2	4	Basal	6.0	10.7	84	0.28
3	4	Basal+0.2% L-methionine	0.5	9.3	87	0.34
4	4	Basal+0.2% L-methionine	6.0	11.8	122	0.37
5	4	Basal+0.2% D-methionine	0.5	8.8	76	0.30
6	4	Basal+0.2% D-methionine	6.0	13.1	129	0.35
7	4	Basal+0.24% OH-methionine[4]	0.5	7.4	67	0.32
8	4	Basal+0.24% OH-methionine	6.0	11.1	123	0.39
		Experiment 2[5]				
9	5	Basal (12% soy protein)[6]	0.5	9.3	32±2	0.13
10	5	Basal	1.0	11.5	43±2	0.14
11	5	Basal	6.0	12.0	55±3	0.17
12	5	Basal+0.1% L-methionine	0.5	12.9	72±2	0.21
13	5	Basal+0.1% L-methionine	1.0	13.9	95±3	0.25
14	5	Basal+0.1% L-methionine	6.0	15.0	119±3	0.30
15	5	Basal+0.2% L-methionine	0.5	13.5	81±2	0.23
16	5	Basal+0.2% L-methionine	1.0	14.9	115±3	0.29
17	5	Basal+0.2% L-methionine	6.0	15.4	135±4	0.32
18	5	Basal+0.1% D-methionine	0.5	12.9	52±3	0.16
19	5	Basal+0.1% D-methionine	1.0	14.1	96±3	0.26
20	5	Basal+0.1% D-methionine	6.0	14.1	117±4	0.31
21	5	Basal+0.2% D-methionine	0.5	13.1	92±2	0.26
22	5	Basal+0.2% D-methionine	1.0	14.4	100±3	0.26
23	5	Basal+0.2% D-methionine	6.0	14.0	133±4	0.35

[1] Pyridoxine hydrochloride.
[2] Average ± standard error of the mean.
[3] Six rats were used per group; rats depleted for 7 days with a pyridoxine-free 20% casein diet before starting on experiment. Average initial weight after depletion was 48 gm.
[4] Calcium α-hydroxy-1-methyl-mercaptobutyric acid; amount added calculated to be equal to that of the methionine used.
[5] Four rats used per group; rats depleted for 10 days with a pyridoxine-free 20% casein diet before starting on experiment. Average initial weight after depletion was 63 gm.
[6] Drackett Alpha Soy Protein, extracted with water and hot methanol.

TABLE 5

Effect of vitamin B_6 on the utilization of valine by weanling rats[1] *(amino acid mix basal diet)*

Group no.	Basal diet no.	Diet	Vitamin B_6 supplement[2]	Av. daily food intake	Av. weight gain in 3 weeks[3]	Av. gain/gm of food intake
			mg/kg diet	*gm*	*gm*	*gm*
1	6	Basal[4]+0.4% L-valine	0.75	7.4	53±3	0.34
2	6	Basal +1.0% D-valine	0.75	3.3	1±1	0.01
3	6	Basal +2.0% D-valine	0.75	7.2	43±4	0.28
4	6	Basal +1.0% D-valine	6.0	6.0	16±2	0.13
5	6	Basal +2.0% D-valine	6.0	6.0	66±3	0.39
6	6	Basal +0.7% L-valine	6.0	10.8	74±3	0.33

[1] Four rats used per group; rats depleted for 10 days with a pyridoxine-free 20% casein diet before starting on experiment. Average initial weight after depletion was 61 gm.
[2] Pyridoxine hydrochloride.
[3] Average ± standard error of the mean.
[4] The basal diet contained an amino acid mix in place of intact protein.

and low pyridoxine (0.5 mg per kg of diet), however, D-methionine was somewhat more active than L-methionine when fed in the soy protein diet (experiment 2). This may be a reflection of a toxic effect as excess methionine in the presence of low pyridoxine may depress growth (De Bey et al., '52). Supplementary experiments (data not presented) indicated that L-methionine at high levels (1% of the diet) depressed growth of weanling rats somewhat more than comparable levels of D-methionine. Similar observations have been reported by Wretlind and Rose ('50). In the present studies, however, the two isomers of methionine at the levels used (0.1 or 0.2% of the diet) permitted equal growth at the high levels of pyridoxine (6 mg per kg of diet) and is in agreement with the reports of other investigators (Wretlind and Rose, '50). Also, the hydroxy analogue of methionine (calcium α - hydroxy-methyl-mercaptobutyric acid) permitted, in the presence of adequate pyridoxine, growth comparable to L- or D-methionine, but was inferior to each when tested at the low level of pyridoxine. In the above studies, efficiency of gain was improved whenever the *level of pyridoxine or methionine in the diet was increased.*

The influence of pyridoxine on the activity of L- and D-valine for the growth of weanling rats may be observed from the results presented in table 5. Animals were fed a protein-free diet that contained an amino acid mixture free of D-leucine (Wretlind, '56; Sauberlich, '61) and controlled amounts of valine and pyridoxine hydrochloride. The activity of D-valine was also observed to be enhanced markedly by the presence of high levels of pyridoxine in the diet. At the low level of pyridoxine (0.75 mg per kg of diet), 1.0% of D-valine permitted essentially no growth of the animals, whereas a level of only 0.4% of L-valine permitted a gain in weight of 53 gm in three weeks. Increasing the level of pyridoxine or D-valine in the diet enhanced the gains obtained with D-valine. Even with ample amounts of pyridoxine in the diet, however, D-valine was considerably inferior to a comparable level of L-valine in permitting growth of rats.

DISCUSSION

Results of the present studies indicate that when a diet is limiting in both pyridoxine and in an essential amino acid (tryptophan or methionine), growth of the rat can be improved, within limits, by supplements of either the vitamin or the amino acid. Furthermore, the results indicate that the activity of the unnatural D-isomer as well as of the L-isomer of essential amino acids may be influenced by the concentration of pyridoxine in the diet and tissues. It was observed that the growth-promoting activities of both L- and D-tryptophan, L- and D-methionine, and D-valine were enhanced with increased amounts of pyridoxine in the diets. The degree of replacement of the natural L-isomer by the D-form appeared to be related to the individual amino acid considered and may be dependent upon the ease and efficiency of the stereonaturalization and the associated transamination, deamination, or amination activities that may be involved. High levels of pyridoxine in the diet could enhance, through association with such activities, the conversion of the D-isomer of certain amino acids to the natural L-form, and as well, spare more of the L-form for protein synthesis and growth.

All aspects involved in the conversion of the D-isomer of amino acids to the corresponding L-isomer are not entirely certain. In general it is considered that in animals the D-isomer is deaminated by D-amino acid oxidases to the corresponding keto acid and then reaminated again by transaminase enzymes to produce the L-form of the amino acid. Since vitamin B_6 is essential for the activation of the transaminase system, a deficiency of pyridoxine in the diet could result in a reduction in the conversion of the D-isomer of amino acids to the natural L-form. Thus, since α-ketoisovaleric acid results in the same quantitative growth in rats as L-valine (Wretlind, '52a), it is quite conceivable that vitamin B_6 enhances the stereonaturalization of D-valine through effects on the α-keto intermediate. The presence of D-amino acids in the diet has been shown to interfere in certain instances in the stereonaturalization of several D-amino acids (Wretlind, '52b, '56). It is possible

that pyridoxine may modify such interferences to permit an improved conversion.

Berezov ('53a) made a study of the urinary products obtained after a test dose of L- or D-tryptophan given to vitamin B_6-deficient rats. It was concluded that vitamin B_6-deficiency reduced the inversion of D-tryptophan to L-tryptophan as a result of a reduction in the transaminase activity. The transaminase system was essential for the reamination of the indolepyruvic acid formed from the D-tryptophan by D-amino acid oxidase to give rise to L-tryptophan. Berezov ('53b) also reported that vitamin B_6-deficiency did not interfere in the formation of urea from glycine and aspartic acid but did reduce it from glutamic acid and associated the differences with relationship to transaminase participation. Beaton et al. ('50) in a study on amino acid metabolism in the rat, suggested that deprivation of pyridoxine did not impair transamination, but delayed deamination. Other investigators have reported that transaminase activities in tissues and serum were reduced in vitamin B_6-deficient rats (Ames et al., '47; Marsh et al., '55; Hsu et al., '58; Babcock, '59; Brin et al., '60).[5]

Armstrong et al. ('50) observed that kidney homogenates from rats deficient in vitamin B^6 were less active in D-amino acid oxidase activity than kidney homogenates from control animals. The relationship of vitamin B_6 is not clear, since D-amino acid oxidase is considered a flavoprotein enzyme. Nitrogen utilization by the deficient animals was also reduced by additions of D-amino acids. DeBey et al. ('52) observed that the addition of DL-methionine to diets fed vitamin B_6-deficient rats produced a growth-depression that was reversed by vitamin B_6. Similar observations have been made in this laboratory.

Vitamin B_6 could also exert an effect on the absorption of amino acids from the intestine or on their excretion into the urine. Akedo et al. ('60) recently presented evidence that the L-isomers of amino acids are actively transported during absorption under the influence of vitamin B_6. The D-isomers appeared to be transferred by simple diffusion independent of any effect from vitamin B_6, however. In pyridoxine deficiency, increased amounts of amino acids have been observed in the urine of rats and mice (Sauberlich and Baumann, '49). The increases were moderately small, however, even with animals exhibiting a pronounced vitamin B_6 deficiency state.

Sure and Easterling ('49) noted an influence of pyridoxine on the improvement of food utilization and protein synthesis. Other investigators have also presented evidence that vitamin B_6 functions in protein synthesis (Lichstein, '56) and in the metabolism of both tryptophan (Sebrell and Harris, '54) and methionine (De Bey et al., '52), and certain other amino acids (Snell, '53). Adequate levels of pyridoxine in the diet could, through association with various enzymatic activities, permit a greater portion of a limiting amino acid to be incorporated into proteins for growth and protect against catabolic losses.

In a similar manner, the degree of replacement of the natural L-isomer of amino acids by the D-form could be enhanced. With increased amounts of vitamin B_6 in the diet, more of the D-forms may be protected from oxidative systems for conversion to the essential L-analogue. Studies of Hawkins et al. ('59) pointed to an increased oxidation of amino acids in rats deprived of vitamin B_6 and fed a diet containing 40% of casein. Their results are in harmony with the observations of others that rats have a high blood urea (Hawkins et al., '46; Beaton et al., '53a, b) and an increased production of urea by the liver (Caldwell and McHenry, '53) in vitamin B_6 deficiency. In pyridoxine-deficient rats there is also an increased excretion of nitrogen (Beaton et al., '53a).

Thus, it would appear that when an amino acid is limiting, added amounts of vitamin B_6 in the diet may have a "sparing-effect" by permitting a greater quantity of the limiting amino acid to be channeled into protein synthesis and growth instead of being channeled into oxidative systems and lost in the urine as urea or as the free amino acid. If additional quantities of the limiting amino acid are added

[5] Brin, M., and M. Tai 1958 Pyridoxine deficiency and serum transaminases. Federation Proc., *17:* 472 (abstract).

instead of vitamin B_6, improved growth can also be obtained by overcoming the otherwise increased loss of the amino acid.

SUMMARY

The influence of dietary supplements of pyridoxine on the activities of the L- and D-isomers of tryptophan, methionine, and valine for the growth of weanling rats was investigated. Diets were designed to be low in pyridoxine and the amino acid under investigation.

Growth and efficiency of gains of the animals were improved by increased levels in the diets of either pyridoxine or the amino acid under study or both.

The growth-promoting activities of both the L- and D-isomers of the amino acids studied were enhanced with increased amounts of pyridoxine in the diets. With an optimum amount of vitamin B_6 in the diet, D-methionine and the hydroxy analogue of methionine were equal to L-methionine in promoting growth; D-tryptophan was about 50% as active as L-tryptophan; D-valine was less than one-third as effective as L-valine.

Dietary pyridoxine levels were observed to influence the pattern of plasma free-amino acids.

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Growth of Rats Fed Protein-Free Diets Supplemented with Purified Amino Acid Mixtures[1,2]

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Considerable attention has been given in this laboratory to the effects of amino acid imbalances on experimental animals. Reports have been made previously on the development of amino acid imbalances in the rat or mouse of tryptophan, threonine, methionine and isoleucine (Sauberlich and Salmon, '55; Sauberlich, '56). Attempts to produce imbalances of additional amino acids by use of intact proteins in diets met with little success, however. Moreover, certain other possible amino acid imbalances were not studied because of an inability to devise suitable diets with respect to amino acid content with the use of readily available natural proteins. In order to control more rigorously and with greater flexibility the amino acid content of diets in an attempt to produce additional amino acid imbalances, consideration was given to the use of diets in which intact protein was completely replaced by amino acid mixtures.

Numerous investigators have studied the growth of rats fed purified amino acid diets and have noted various difficulties with respect to acceptance, adaptation, optimum growth, antagonisms, imbalances and other effects (Maddy and Swift, '52; Ramasarma et al., '49; Rose et al., '48; Russell and Taylor, '48; Phillips and Berg, '54; Koeppe and Henderson, '55; Allison, '55; Wretlind, '56; Rama Rao et al., '60a, b; Hepburn et al., '60; Calhoun et al., '60). The majority of these studies have been included and discussed in the reviews of Elvehjem and Krehl ('55), Elvehjem and Harper ('55), Elvehjem ('56), Harper ('56, '58a) and Salmon ('58). The literature is also complexed by the numerous and various amino acid "unbalances," "imbalances," "deficiencies" and "toxicities" reported and as reviewed by Harper ('58a).

The initial portion of the present report is concerned with the development of diets in which intact protein has been replaced by amino acid mixtures. The resulting diets were then used in several amino acid imbalance studies. In the present study, the severity of a specific partial amino acid deficiency (or limiting amino acid) has been increased by providing in the diet a supplementary amount of a mixture of all of the amino acids other than the limiting one. In this manner, imbalance deficiencies of histidine, isoleucine, leucine, lysine and valine were demonstrated.

EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain were placed individually in wire-bottomed cages in an air-conditioned room. Food and water were given ad libitum and the animals were weighed twice a week. Food consumption records were kept in most instances. Composition of the basal diets used is given in table 1. Composition of the various amino acid mixtures incorporated into the diets is presented in table 2. Amino acids were

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TABLE 1

Composition of experimental diets (gm/kg of diet)

	Diet no.[1]										
	1	2	3	4	5	6	7	8	9	10	11
Casein (extracted)	—	—	100	200	—	—	—	—	—	—	200
Dextrin	( )[2]	( )[2]	( )[3]	( )[3]	392.5	435.5	354.0	519.7	265.1	442.5	447
Sucrose	120	120	120	120	120	120	120	120	120	120	120
Cellulose[4]	20	20	20	20	20	20	20	20	20	20	20
Salts[5]	50	50	50	50	50	50	50	50	50	50	50
Cod liver oil	10	10	10	10	10	10	10	10	10	10	10
Corn oil	40	—	(40)[3]	(40)[3]	—	—	—	—	—	—	—
Lard	—	100	(100)[3]	(100)[3]	150	150	150	150	150	150	150
L-Cystine	—	—	3	3	—	—	—	—	—	—	—
Choline	2	2	2	2	2	2	2	2	2	2	2
Inositol	1	1	1	1	1	1	1	1	1	1	1
Aureomycin, mg[6]	100	100	100	100	100	100	100	100	100	100	100
Folacin, mg	2	2	2	2	2	2	2	2	2	2	2
Vitamin B_{12}, μg	50	50	50	50	50	50	50	50	50	50	50
Amino acid mixture: (see table 2 for composition)											
No.	(A–F)	(A–F)	—	—	G	H	I	G	G	J	—
Amount, gm (see table 2 for weight of mixture added			—	—	254.5	211.5	293.0	127.3	381.9	204.5	—

[1] All diets were supplemented with the following vitamins (mg/kg of diet): α-tocopherol, 100; α-tocopheryl acetate, 100; 2-methyl-1, 4-naphthoquinone, 5; niacin, 25; Ca pantothenate, 30; thiamine, 6; riboflavin, 6; pyridoxine, 12; and biotin, 0.5.

[2] Dextrin was added to the diets to make a final weight of one kilogram; the amount of dextrin added was dependent upon the amino acid mixture used.

[3] Corn oil and lard were used interchangeably as indicated in following tables; dextrin (Will Corporation, New York) was added to the diets to make a final weight of a kilogram; supplements were added at the expense of dextrin.

[4] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[5] Salmon ('47).

[6] Aureomycin (chlortetracycline); Lederle Laboratories, Pearl River, New York.

TABLE 2

Composition of purified amino acid mixtures used in the various rat diets

	Amino acid mixture									
	A	B	C	D	E	F	G	H	I	J
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
L-Alanine	—	—	—	—	—	—	—	6.0	—	—
DL-Alanine	4.0	6.0	6.0	20.0	5.0	25.0	6.0	—	6.0	6.0
L-Arginine·HCl	5.0	8.0	8.0	8.0	4.8	8.0	8.0	8.0	8.0	8.0
L-Aspartic acid	—	6.0	6.0	—	3.3	—	6.0	6.0	—	6.0
DL-Aspartic acid	—	—	—	—	—	—	—	—	6.0	—
L-Asparagine·H_2O	2.0	6.0	6.0	5.0	3.0	—	6.0	6.0	—	6.0
DL-Asparagine·H_2O	—	—	—	—	—	—	—	—	6.0	—
L-Cystine	2.0	3.0	3.0	3.0	3.4	3.0	3.0	3.0	3.0	3.0
L-Glutamic acid	20.0	36.0	40.0	5.0	21.6	5.0	40.0	40.0	40.0	40.0
Glycine	1.0	7.0	4.0	5.0	2.0	5.0	4.0	4.0	4.0	4.0
L-Histidine·HCl	8.6	7.0	10.0	8.0	3.8	8.0	10.0	10.0	10.0	10.0
L-Leucine	12.0	20.0	20.0	20.0	10.0	20.0	20.0	20.0	—	—
DL-Leucine	—	—	—	—	—	—	—	—	40.0	—
L-Isoleucine	—	—	—	—	—	—	—	15.0	—	—
DL-Isoleucine	20.0	30.0	25.0	30.0	16.0	30.0	30.0	—	30.0	—
L-Lysine·HCl	15.0	18.5	17.0	18.5	10.0	17.0	18.5	18.5	—	18.5
DL-Lysine·HCl	—	—	—	—	—	—	—	—	37.0	—
L-Methionine	—	—	—	—	3.5	—	—	8.0	—	—
DL-Methionine	8.0	8.0	8.0	8.0	—	8.0	8.0	—	8.0	8.0
L-Phenylalanine	—	—	—	—	—	—	—	6.5	—	—
DL-Phenylalanine	12.0	13.0	12.0	13.0	7.0	13.0	13.0	—	13.0	13.0
L-Proline	2.0	8.0	5.0	1.0	8.2	—	5.0	5.0	5.0	5.0
L-Serine	—	—	—	—	—	—	—	5.0	—	—
DL-Serine	2.0	10.0	5.0	2.0	6.8	—	5.0	—	5.0	5.0
L-Threonine	—	—	—	—	—	—	—	7.5	—	—
DL-Threonine	14.3	18.0	15.0	18.0	9.0	15.0	15.0	—	15.0	15.0
L-Tryptophan	—	—	—	—	1.4	—	—	5.0	—	—
DL-Tryptophan	4.0	5.0	4.0	5.0	—	4.0	5.0	—	5.0	5.0
L-Tyrosine	6.0	10.0	8.0	5.0	6.4	5.0	8.0	8.0	8.0	8.0
L-Valine	—	—	—	—	—	—	—	14.0	—	—
DL-Valine	20.0	25.0	28.0	25.0	15.0	25.0	28.0	—	28.0	28.0
$NaHCO_3$	12.7	14.7	15.4	15.2	8.0	14.5	16.0	16.0	16.0	16.0
Total weight, gm	170.6	259.2	245.4	214.7	148.2	205.5	254.5	211.5	293.0	204.5

finely ground, passed through a 20-mesh screen, and well mixed prior to use. The composition of the mixtures as well as of the diets was developed with consideration to certain principles of protein and amino acid metabolism. Factors considered included requirements for specific amino acids, influence of D-isomers and "nonessential" amino acids, energy and nitrogen level of the diet and association of other dietary components with protein and amino acid metabolism (Allison, '55).

Several of the mixtures were devised to simulate in part the amino acid composition of certain proteins. Thus, mixture B was designed with consideration to the amino acid composition of egg albumin and lactalbumin; mixture E simulated the amount of amino acids furnished by 10% of casein in a diet, with added cystine. Amino acid mixture A was essentially, that used by Maddy and Swift ('52) and similar to that of Wretlind ('56). Mixture C was devised with respect to the relative needs of the rat for amino acids and also with consideration to the activity and cost of the isomers of the individual amino acids. Mixtures D and F were devised to be low in glutamic acid and the "nonessential" amino acids, but complemented with a high level of alanine to compensate for the change in nitrogen content. Mixture G was the same as mixture C, except that the amounts of DL-isoleucine, L-lysine, DL-phenylalanine and DL-tryptophan were increased for better balance. Mixture H was composed of only the L-isomers of the amino acids at the same level as furnished by mixture G. Mixture I was composed of these amino acids in the DL-form that were readily available, with the amounts used adjusted to compensate for the inactivity of certain D-isomers. Amino acid mixture J was devoid of leucine and isoleucine and was used in certain imbalance studies (see table 5). Except for mixture I, L-leucine was used instead of DL-leucine to prevent any possible antagonistic effects of the D-isomer (Wretlind, '56).

Where used, the D-isomers of tryptophan and methionine were considered equal to that of the corresponding L-forms. Asparagine was used in place of aspartic acid or to replace a portion of it in the amino acid mixtures in order to minimize the possible observed toxic effects of aspartic acid.[4] Hydroxyproline was omitted from all diets for similar reasons. Sodium bicarbonate was added to each mixture in an amount sufficient to buffer the hydrochloric acid contributed by several amino acids.

Dextrin, cellulose and Aureomycin (chlortetracycline) were added to the diets as possible aids for more favorable absorption of the diet and maintenance of the intestinal flora. An added allowance of pyridoxine was made for possible assistance in the metabolism of the abnormal amounts of D-amino acids present in the diets. In a few instances, various levels of corn oil and lard were used in the diets at the expense of dextrin.

In certain instances, specific amino acids were omitted from the amino acid mixtures and controlled amounts of the omitted amino acids were then added to the respective diets (see tables and text). The diets were stored in glass jars in a refrigerator and re-mixed fresh every 10 to 14 days. No difficulties were encountered in the mixing, storage or feeding of the purified amino acid diets.

At the termination of several experiments, blood samples were obtained for plasma free-amino acid analyses and livers removed for determination of fat content.

RESULTS

Growth of rats fed protein-free diets supplemented with purified amino acid mixtures

The objective of these studies was to replace the protein in experimental diets with a mixture of amino acids that would permit normal growth of weanling rats. The major results are presented in table 3. In experiment 1, amino acid mixtures of various compositions were compared in the presence of lard or corn oil. The various amino acid mixtures supported, in each instance, at least a 50-gm gain in weight of the rats for the initial two-week period. Mixtures B and C, however, were superior to the others tested. Results indicated that for rapid early growth it was necessary that some of the "nonessential"

[4] Sauberlich, unpublished data.

TABLE 3

Growth of weanling rats fed protein-free diets supplemented with amino acid mixtures[1]

Group no.	Basal diet no.	Diet	Av. daily food intake	Av. gain/gm food intake	Av. gain in weight at[2]	
					2 weeks	3 weeks
			gm	*gm*	*gm*	*gm*
		Experiment 1[3]				
1	1	Amino acid mix A, 4% corn oil	11.8	0.32	53	—
2	2	Amino acid mix A, 10% lard	11.4	0.32	51	—
3	2	Amino acid mix B, 10% lard	11.2	0.52	81	120±3
4	2	Amino acid mix C, 10% lard	11.9	0.50	83	125±4
5	1	Amino acid mix D, 4% corn oil	10.5	0.43	63	—
6	2	Amino acid mix D, 10% lard	10.9	0.46	70	—
7	2	Amino acid mix E, 10% lard	12.7	0.36	65	—
8	1	Amino acid mix F, 4% corn oil	11.0	0.38	59	—
9	2	Amino acid mix F, 10% lard	11.3	0.39	62	—
10	3	10% casein 4% corn oil	14.0	0.37	73	113±4
11	3	10% casein 10% lard	14.1	0.34	69	105±3
12	4	20% casein 4% corn oil	13.7	0.47	85	126±3
13	4	20% casein 10% lard	11.7	0.52	85	129±3
		Experiment 2				
14	5	Regular amino acid mix G	9.2	0.68	81	131±3
15	6	L-Amino acid mix H	9.5	0.65	81	129±2
16	7	DL-Amino acid mix I	8.4	0.56	61	103±4

[1] Four rats used/group; average initial weight was 40 gm in experiment 1 and 44 gm in experiment 2.

[2] Average ± standard deviation.

[3] In experiment 1, food intake and gain per gram of food intake are based on the initial 14-day experimental period.

amino acids be present in the diet. Use of high amounts of alanine or glutamic acid did not substitute satisfactorily for the nonessential amino acids. Growth of animals in group 7, fed amino acid mixture E, simulating a 10% casein diet, approximated that of group 11, fed a diet containing 10% of intact casein supplemented with cystine. The use of corn oil in place of lard in the diet did not appear to influence the results appreciably. No indications of diarrhea or poor food intakes were observed. Diets were readily consumed as may be noted from the tables. Throughout the studies, within respective groups, remarkably little variation in growth was noted between animals.

Amino acid mixture C permitted growth approximating that obtained with a regular 20% casein control diet supplemented with L-cystine (table 3). Additional minor changes in amino acid mixture C resulted in mixture G, which was used in the subsequent experiments (diet 5). In experiment 2 (group 14; table 3) weanling rats weighing an average of 44 gm gained over 40 gm per week during a three-week experimental period when fed diet 5 containing amino acid mixture G. Even during the initial week of receiving the diet, without any adjustment period, the animals gained approximately 5 gm per day. Diet 5, containing amino acid mixture G, has been used in several subsequent studies in this laboratory and has always permitted growth of weanling rats equal to or exceeding that obtained with a 20% casein diet.

Since the above amino acid mixtures contained some DL-amino acids, the possibility existed that the D-forms may have depressed growth. When amino acid mixture H containing only L-isomers was fed, however, the growth obtained was virtually identical to that observed with amino acid mixture G (table 3; group 14 vs. 15). The present cost of certain L-amino acids would preclude the general use of mixture H. Growth of rats fed diets containing the DL-amino acid mixture (mixture I) was somewhat depressed when compared with animals fed amino acid mixtures G and H, although gains of over 30 gm per week were still obtained.

Subsequent studies[5] have indicated that the dextrin and cellulose could be replaced with sucrose, the Aureomycin omitted, and normal levels of pyridoxine used without any marked influence on the growth of rats fed the amino acid mixtures. A level of 15% of lard in the diet proved to be a more satisfactory level than 5 or 10% from the standpoint of mixing and feeding of the diet as well as of a possible slight effect on growth.

In supplementary studies, the liver fat contents and the levels of plasma-free amino acids of rats fed the diets containing the optimum amino acid mixtures as substitutes for protein approximated those of animals fed a diet containing 20% of casein or a stock diet. Moderately fatty livers (20 to 25% fat on dry basis), however, were observed in rats when fed diets containing the amino acid mixture at a level one-half of that normally used.

Studies on amino acid imbalances in the rat with the use of amino acid mixtures

In table 4 are presented data on the production of amino acid imbalances related to lysine, isoleucine, valine, leucine and histidine. The basal diet used to produce these imbalances contained amino acid mixture G at one-half the level used in the previous experiments (diet 8 vs. 5). The amino acid under study was further reduced in the diet so that excess amounts would not be present. When the amino acids in the diet were then doubled or tripled, except for the amino acid under study, growth depressions or imbalances were observed (namely, groups 7, 8 and 9; table 4). The imbalances thus created, however, were corrected by increasing the level in the diet of the amino acid under study to its normal ratio with respect to

[5] Sauberlich, unpublished data.

TABLE 4

Effect of certain amino acid imbalances on the growth of weanling rats[1]

Group no.	Basal diet no.	Diet and level of the amino acid controlled[1]	Av. daily food intake	Av. gain/gm of food intake	Av. gain in 3 weeks
		gm/kg of diet	*gm*	*gm*	*gm*
1	8	10% Amino acids; 4.5 gm L-lysine·HCl	8.2	0.22	39
2	5	20% Amino acids; 4.5 gm L-lysine·HCl	6.6	0.20	28
3	9	30% Amino acids; 4.5 gm L-lysine·HCl	6.6	0.15	19
4	8	10% Amino acids; 10.0 gm DL-isoleucine	8.9	0.24	45
5	5	20% Amino acids; 10.0 gm DL-isoleucine	7.8	0.33	54
6	9	30% Amino acids; 10.0 gm DL-isoleucine	5.7	0.20	18
7	8	10% Amino acids; 8.0 gm DL-valine	9.2	0.30	54
8	5	20% Amino acids; 8.0 gm DL-valine	6.9	0.15	24
9	9	30% Amino acids; 8.0 gm DL-valine	4.2	0.14	13
10	8	10% Amino acids; 6.0 gm L-leucine	9.2	0.30	57
11	9	30% Amino acids; 6.0 gm L-leucine	6.7	0.18	28
12	8	10% Amino acids; 2.5 gm L-histidine·HCl	9.7[2]	0.35[2]	44[3]
13	5	20% Amino acids; 2.5 gm L-histidine·HCl	8.9[2]	0.24[2]	27[3]
14	9	30% Amino acids; 2.5 gm L-histidine·HCl	9.1[2]	0.17[2]	18[3]
15	8	10% Amino acids; all normal ratios	9.3	0.31	66
16	5	20% Amino acids; all normal ratios	9.4	0.49	96
17	9	30% Amino acids; all normal ratios	9.6	0.51	112

[1] The basal diets contained the purified amino acid mixture G at approximately the levels indicated (see table 1). However, in groups 1–14, the amino acid indicated was omitted from the mix and added to the respective diets at the levels indicated. In groups 15–17, all amino acids were present at the normally employed ratios as indicated in amino acid mixture G. Four rats were used per group; average initial weight was 51 gm.

[2] Calculated on the final 14-day experimental period.

[3] Average weight gain in two weeks for groups 12, 13, 14.

the other amino acids (groups 15, 16, and 17).

The possibility of an antagonism between single amino acids was investigated in another series of experiments, using leucine and isoleucine as an example (table 5). For part of the groups of animals, the level of isoleucine in the diets was kept constant while the level of leucine was increased. As the level of leucine was increased, a growth depression occurred (groups 2 to 6). The depression in growth could be readily prevented by increasing the level of isoleucine in the diet, even when the level of leucine fed was twice that normally used. Growth of the animals fed the balanced amino acid mixtures exceeded somewhat that obtained with a 20% casein control diet (group 1 vs. 2). Thus, in this instance an imbalance or antagonism was produced by an excess in the purified amino acid-mix diet of a single amino acid and the condition could be corrected by supplements of a single amino acid. Similar studies with various combinations of isoleucine, leucine and valine were met with variable results and need further investigation before definite conclusions can be stated. An increase of 100% in the amount of isoleucine in the diet, however, did not produce a growth-depression in rats fed limiting amounts of leucine (groups 7 and 8; table 5).

DISCUSSION

The concept of amino acid balance in the diet in order to prevent growth depressions from amino acid imbalances or antagonisms is evident again from the results of these studies. In previous investigations intact protein was used to produce imbalances of tryptophan, methionine, threonine and isoleucine (Sauberlich and Salmon, '55; Sauberlich, '56; Harper, '58a, b; Salmon, '58). The use of amino acid mixtures has permitted the demonstration of additional imbalances and emphasizes further the general possible occurrence of the imbalance phenomenon. Similarly, it should be noted again that the amino acid requirements of the rat are not constant factors, but are related to the diet used and, in particular, to the amino acid balance and protein or nitrogen level of the diet. Thus, reports that the requirements for individual amino acids increase with increasing protein intake (Allison, '55; Bressani and Mertz, '58; Harper, '58a, b) may well be related to amino acid imbalances and antagonisms. The general phenomenon and occurrence of the amino acid imbalances and related conditions has been previously discussed (Salmon, '54, '58; Elvehjem and Harper, '55; Harper, '56; Sauberlich and Salmon, '55; Sauberlich, '56) and intensively reviewed (Elvehjem and Krehl, '55; Harper, '58a).

TABLE 5

Effect of leucine and isoleucine balance in the diet on the growth of weanling rats[1]

Group no.	Basal diet no.	Diet and level of the amino acids controlled[2]	Av. daily food intake	Av. weight gain in 3 weeks	Av. gain/gm of food intake
		% of diet	*gm*	*gm*	*gm*
1	11	Casein, 20%	11.7	101.4	0.41
2	10	Basal + 2.0% L-leucine + 3.0% DL-isoleucine	14.2	116.0	0.39
3	10	Basal + 0.5% L-leucine + 0.5% DL-isoleucine	7.8	22.3	0.14
4	10	Basal + 2.0% L-leucine + 0.5% DL-isoleucine	6.5	−0.4	—
5	10	Basal + 4.0% L-leucine + 0.5% DL-isoleucine	6.6	−7.6	—
6	10	Basal + 4.0% L-leucine + 6.0% DL-isoleucine	13.7	117.4	0.41
7	10	Basal + 0.5% L-leucine + 3.0% DL-isoleucine	8.0	37.6	0.22
8	10	Basal + 0.5% L-leucine + 6.0% DL-isoleucine	8.3	40.7	0.23

[1] Four rats used per group; average initial weight 58 gm.

[2] Basal diet 10 contained purified amino acid mixture J, with L-leucine and DL-isoleucine controlled to the levels indicated. Normal levels of leucine and isoleucine are those as used with group 2, which is equivalent to basal diet 5 (regular amino acid mixture diet). Diet 11 was not supplemented with L-cystine.

Although amino acid mixtures as substitutes for protein in the diet may have limited immediate practical application, their usefulness for studies on amino acid interrelationships, antagonisms, and imbalances has been indicated in the present study. Greater flexibility is available in altering the amino acid content of a diet with such mixtures. Thus, for example, the antagonism of isoleucine by leucine could be readily observed by such alterations. This antagonism is similar to the condition observed by Sauberlich ('56) and Harper et al. ('54, '55) and Benton et al. ('56).

It appears that normal growth of weanling rats may be obtained when properly balanced amino acid mixtures are used as substitutes for protein in experimental diets. This suggests that proteins, from the standpoint of nutrition, probably do not possess, other than the amino acids they contribute, any inherent special properties or components. Previous studies on amino acid mixtures as a nitrogen source for the young rat have indicated less than optimum growth rates (Russell and Taylor, '48; Rose et al., '48; Ramasarma et al., '49; Maddy and Swift, '52). This has led to the speculation that other factors, such as imbalances, toxic effects of D-amino acids, peptides and growth factors, are responsible for these differences (Woolley, '46; Womack and Rose, '46; Berg, '53). For example, Maddy and Swift ('52) noted that the addition of either streptomycin or Aureomycin to the amino acid diet produced a significant increase in the growth rate of rats. Stimulation in growth was comparable to that obtained by the addition of intact casein to the amino acid diet. Addition of monosodium glutamate to the amino acid diet did not improve the growth of rats, whereas, in contrast to the recent findings of Hepburn et al. ('60), additional supplements of glutamic acid depressed growth. Present observations indicate that the level and balance or ratio of the amino acids in the diet are of major importance in order to obtain normal growth of young rats. For such optimum growth, consideration must be given to the addition of certain "nonessential" amino acids to the diet to serve as a supplemental source of nitrogen and to furnish those amino acids that cannot be synthesized by the animal at a sufficiently rapid rate to permit optimum growth. Evidence for such a consideration has been presented also by Rose et al. ('48), Allison ('55) and Rechcigl et al. ('57), and by the recent studies of Hepburn et al. ('60) and Calhoun et al. ('60). It is recognized, however, that in some instances the influence of certain D-amino acids may be important (Phillips and Berg, '54; Wretlind, '56; Wachter and Berg, '60).

SUMMARY

1. Weanling rats gained approximately 40 gm per week when fed amino acid diets, devoid of protein, which was equal to the growth obtained with a 20% intact casein control diet. "Nonessential" amino acids appeared to be necessary in the diet in order to obtain an optimum early growth rate. In general, the D-isomers of amino acids did not cause an appreciable interference in growth.

2. Growth of the animals was related to the balance or ratio of the individual amino acids in the amino acid mixture or diet. Imbalance deficiencies of histidine, isoleucine, leucine, lysine and valine were demonstrated with the use of the amino acid mixtures. Isoleucine was antagonized by the leucine content of the diet.

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Nutrition of Salmonoid Fishes

IX. CARBOHYDRATE REQUIREMENTS OF CHINOOK SALMON

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Previous studies with fish have demonstrated that trout are capable of absorbing a large percentage of the carbohydrate supplied in the diet (Phillips et al., '48). The rate of carbohydrate absorption in fish, however, is much slower than that in warm blooded animals (Cori, '25). Phillips et al. ('48) also reported that trout were incapable of excreting large quantities of carbohydrate in their urine, and consequently, that high dietary carbohydrate levels cause growth inhibition, pathological glycogen deposition and death. These workers concluded that (digestible) carbohydrate levels in excess of 9 to 12% (wet weight) of the diet were toxic to trout. McLaren et al. ('46) confirmed these observations in studies on rainbow trout fed purified diets containing increasing concentrations of glucose. The diets with higher carbohydrate levels (and correspondingly lower protein and vitamin levels) produced less fish growth, enlarged livers and greater mortalities. These workers first concluded that carbohydrate intakes greater than 20% were toxic to trout; however, in subsequent studies McLaren et al. ('47) fed more nutritionally adequate diets to rainbow trout and could find no deleterious effects with diets containing as much as 45% carbohydrate.

Since the majority of these studies involved diets in which carbohydrate content was increased at the expense of protein and vitamins, it is not clear whether these workers were indeed studying the effect of high dietary carbohydrate levels or instead, that of protein and/or vitamin deficiencies. Because of the apparent contradictions of the effect of carbohydrate content on the nutrition of fish, additional studies employing otherwise nutritionally adequate diets were necessary. Therefore, experiments have been performed in which Chinook salmon (*Oncorhynchus tshawytscha*) have been fed variations of the well-characterized semi-synthetic diet of Halver ('57) containing varying amounts of carbohydrate, fat and alpha-cellulose. The effect of different carbohydrate sources has also been examined.

EXPERIMENTAL

Actively feeding Chinook salmon fingerlings were raised with a test diet (Halver, '57) from eggs supplied by the Spring Creek National Fish Hatchery, Underwood, Washington, and used throughout the present studies. Feeding trials were conducted in screen-covered plastic sealed wooden troughs furnished with spring water, heated to 10°C and supplied to each trough at the rate of three gallons per minute per trough.

The optimum protein level for Chinook salmon at 10°C water temperature is about 50% of the diet (DeLong et al., '58) and a test diet with this composition has been often used for feeding studies at this laboratory (diet G, table 1). In order to provide more latitude for variations in carbohydrate content of the diets used in the present studies, protein was decreased to a level of 36%. Since this concentration of protein would not provide for maximum fish growth at this water temperature, the test diets were supplemented with arginine and methionine, the indispensable amino acids most limiting in the 36% casein-gelatin mixture for sal-

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TABLE 1

Composition of carbohydrate level diets (exp. 1)

	Diets														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Casein	27	27	27	27	27	27	38	27	27	27	27	27	27	27	27
Gelatin	9	9	9	9	9	9	12	9	9	9	9	9	9	9	9
Arginine·HCl	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
Methionine	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Dextrin	0	10	20	30	40	48	28	0	30	0	0	10	20	30	40
Corn oil	6	6	6	6	6	6	7	14	14	6	12	6	6	6	6
Cod liver oil	2	2	2	2	2	2	2	2	2	2	4	2	2	2	2
Minerals[1]	4	4	4	4	4	4	4	4	4	4	8	4	4	4	4
Vitamins[1]	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1
Alpha-cellulose	48	38	28	18	8	0	8	40	10	0	0	0	0	0	0
CMC[2]	2	2	2	2	2	2	0	2	2	2	4	2	2	2	2
Water	200[3]	200[3]	100	100	100	100	100	100	100	52.5	67.5	62.5	72.5	82.5	92.5
Total	300.5	300.5	200.5	200.5	200.5	200.5	200	200.5	200.5	105	135	125	145	165	185
Cal./100 gm diet[4]	74	88	152	174	195	211	200	147	210	212	218	211	210	212	211
Gm protein/100 gm diet	12.5	12.5	18.7	18.7	18.7	18.7	25.0	18.7	18.7	35.7	27.8	30.0	25.9	22.7	20.2
Mg protein/Cal.	169	142	123	108	96	89	125	128	89	169	128	142	123	107	96

[1] Minerals and vitamin mixtures the same as reported previously (Halver, '57; Coates and Halver, '58).

[2] Carboxymethylcellulose—necessary for proper feeding consistency to diets.

[3] Additional water required for proper feeding consistency.

[4] Calculation for the energy content of the diets based on the assumption that in cal./gm: fat = 9.00, protein = 4.00 and dextrin = 4.23.

mon.[2,3] Methionine and arginine were also found to be the most limiting of the essential amino acids in casein diets for young guinea pigs (Heinicke et al., '55). The composition of the diets used in the present studies is shown in tables 1 and 2. The method of diet preparation and general feeding techniques were the same as those previously reported (Halver, '57).

For the first feeding trial in which diets containing different concentrations of dextrin, fat and alpha-cellulose were fed (table 1), 15 groups of 400 Chinook salmon (average weight 0.65 to 0.70 gm) were hand-counted into experimental hatchery troughs and fed the different diets for 18 weeks. In the second experiment in which 8 different carbohydrate sources were compared (table 2) at the same concentration, 16 groups of 500 Chinook salmon (average weight 0.55 to 0.60 gm) were used and were fed for 14 weeks. The fish were fed a slowly sinking diet expelled through a garlic press or food grater into the forward surface of the water. Each feeding was continued as long as the fish actively accepted food. Fish were fed three times daily on a rigid schedule and a careful record was maintained on the weight of diet fed to each trough. Since previous recovery experiments had demonstrated a maximum food loss of only 5% with this method of feeding, the weight of diet fed to each trough was assumed to represent the actual food consumed by that group of fish. The entire population of each trough was weighed bi-weekly. Troughs were cleaned daily without removing the fish, and were drained, cleaned and disinfected during

[2] Halver, J. E., D. C. DeLong and E. T. Mertz 1959 Methionine and cystine requirements of chinook salmon. Federation Proc., *18*: 527 (abstract).

[3] Halver, J. E. 1960 Vitamin and amino acid requirements of salmon. Proc. Vth International Congress on Nutrition, abstract 191.

TABLE 2

Composition of carbohydrate quality diets (exp. 2)

	Diets							
	C	P	Q	R	S	T	U	V
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Casein	27	27	27	27	27	27	27	27
Gelatin	9	9	9	9	9	9	9	9
Arginine·HCl	1	1	1	1	1	1	1	1
Methionine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Dextrin	20							
Fructose		20						
Galactose			20					
Glucosamine				20				
Glucose					20			
Maltose						20		
Potato starch							20	
Sucrose								20
Corn oil	6	6	6	6	6	6	6	6
Cod liver oil	2	2	2	2	2	2	2	2
Minerals[1]	4	4	4	4	4	4	4	4
Vitamins[1]	1	1	1	1	1	1	1	1
Alpha-cellulose	28	28	28	28	28	28	28	28
CMC[2]	2	2	2	2	2	2	2	2
Water	100	100	100	100	100	100	100	100
Total	200.5	200.5	200.5	200.5	200.5	200.5	200.5	200.5
Cal./100 gm diet[3]	152	148	147	152	148	148	151	150
Gm protein/100 gm diet	18.7	18.7	18.7	18.7	18.7	18.7	18.7	18.7
Mg protein/cal.	123	126	127	123	126	126	124	125

[1] Mineral and vitamin mixtures the same as reported previously (Halver, '57; Coates and Halver, '58).

[2] Carboxymethylcellulose—necessary for proper feeding consistency of diets.

[3] Calculations for energy content of the diets based on the assumption that in cal./gm: fat = 9.00, protein = 4.00, dextrin = 4.23, fructose = 3.75, galactose = 3.72, glucosamine = 4.13, glucose = 3.75, maltose = 3.75, starch = 4.11, and sucrose = 3.96.

the bi-weekly weighing periods. Daily mortalities were recorded and removed immediately from all troughs.

Samples were collected for histological examination from all groups of fish at monthly intervals and also at the end of the experimental period. Histopathological examinations were carried out using the techniques of Wood et al. ('57a). Proximate analysis samples were removed at the start of the feeding studies and from all troughs upon termination of the experiments. Percentage of moisture, protein, lipid and ash were determined by official AOAC methods as described by Wood et al. ('57b). Liver weights were determined at the completion of the feeding studies. Random samples of 20 fish were removed from each trough, fish were stunned individually by an electric current, dried immediately and weighed to the nearest hundredth gram. The liver was rapidly removed from each fish and weighed to the nearest tenth milligram. The percentage contribution of liver to total fish weight could thus be calculated for each fish in the sample. Liver glycogen determinations were performed on all groups of fish upon termination of the feeding experiments. Livers were rapidly removed from a random sample of 20 fish taken from each trough and pooled into two duplicate 10-liver samples in tared tubes containing KOH. Glycogen was determined using a modification (Beatty et al., '59) of the anthrone procedure of Roe ('55).

RESULTS

In experiment 1, 15 groups of 400 Chinook salmon fingerlings were fed a variety of diets containing different concentrations of dextrin, alpha-cellulose and fats (table 1) for an 18-week period. Individual weight gains, mortalities and food consumption of these groups are shown in table 3 and individual group growth curves plotted in figure 1.

As the concentration of dextrin in the diets was increased from zero to 48% and alpha-cellulose concentration simultaneously decreased (diets A–F), the average rate of fish growth also increased. Weight gain remained essentially constant for dextrin levels greater than 20% (diets C–F), however. When alpha-cellulose was omitted from these diets (diets J, L–O), however, the rate of fish growth did not alter appreciably with increasing dextrin concentration. Consequently, it appears that in high cellulose diets the reduced fish growth observed with zero and 10% of dextrin (diets A and B) must result

TABLE 3

Effect of carbohydrate, alpha-cellulose or fat level on rate of growth and food intake of Chinook salmon[1] (exp. 1)

Diet	Av. increase weight/ fish	Gain/fish 0–18 weeks	Cumulative mortalities 0–18 weeks	Cumulative gain/trough[2] 0–18 weeks	Total diet fed/trough 0–18 weeks	Total protein fed/trough 0–18 weeks	Gm gain/ gm protein fed 0–18 weeks	Gm gain/ cal. fed 0–18 weeks
	gm	*gm*	%	*gm*	*gm*	*gm*	*gm*	*gm*
A	0.65	2.39	9.2	752	3650	456	1.65	0.279
B	0.65	3.60	12.0	1144	4282	523	2.19	0.308
C	0.66	4.45	6.8	1562	3523	659	2.37	0.292
D	0.68	4.76	6.0	1593	3637	680	2.34	0.252
E	0.68	4.93	8.0	1707	4044	755	2.26	0.217
F	0.66	5.08	8.3	1778	4187	784	2.27	0.201
G	0.68	4.70	8.5	1563	3560	889	1.76	0.220
H	0.68	2.41	15.8	761	2193	410	1.86	0.236
I	0.67	3.25	15.0	977	2781	520	1.88	0.168
J	0.67	4.50	9.0	1561	2437	869	1.80	0.302
K	0.65	2.79[3]	9.0[3]	890[3]	1389[3]	386[3]	2.31[3]	0.294[3]
L	0.67	4.66	6.5	1624	2879	864	1.88	0.268
M	0.70	4.53	7.3	1419	2853	738	1.92	0.237
N	0.67	4.57	9.7	1505	3331	756	1.99	0.213
O	0.69	4.88	7.0	1621	3864	780	2.08	0.199

[1] 400 fish/trough.
[2] Corrected for mortalities and sample removal.
[3] 14 weeks only.

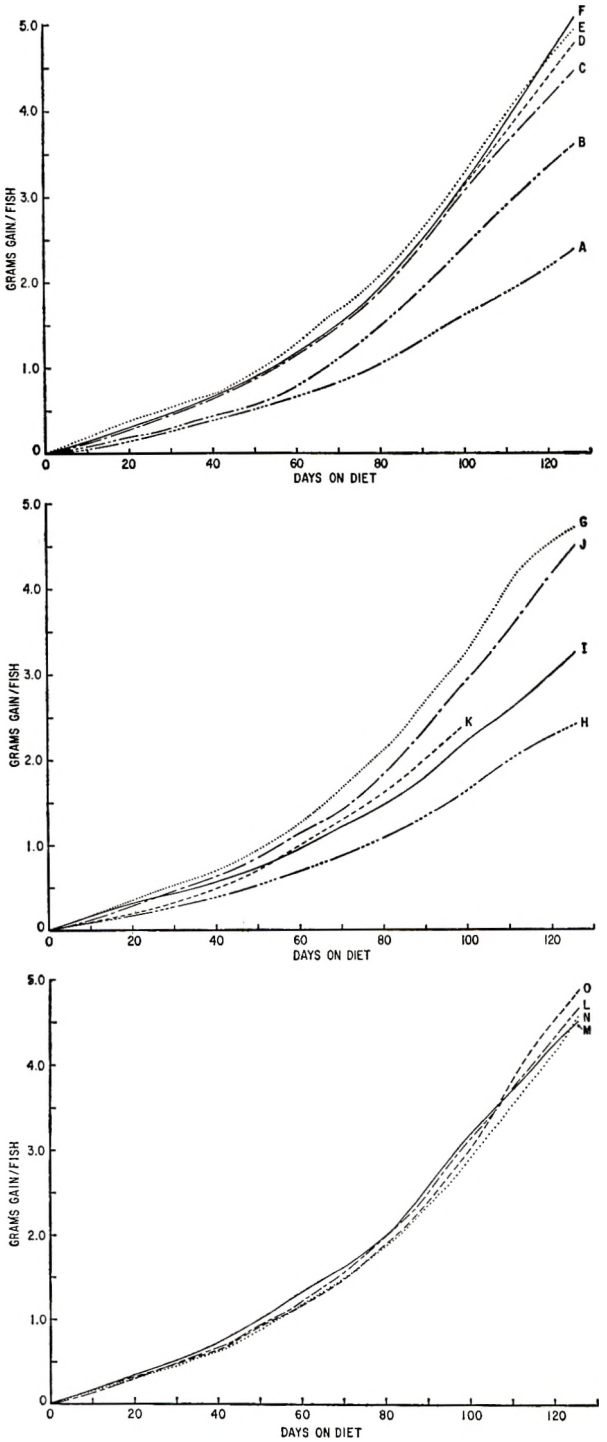

Fig. 1 Weight gain of Chinook salmon fed carbohydrate levels. Upper center and bottom growth curves show respective weight gains of fish fed the carbohydrates and the control diet listed in table 1.

from an inability of the salmon to ingest sufficient nutrients for optimal growth. The total diet consumption data in table 3 confirms this suggestion since the total weight of diet consumed by groups A and B is essentially identical to that consumed by groups C–F. This maximum, presumably determined by the stomach capacity of the test fish, did not permit adequate protein or energy intake with the high alpha-cellulose and low nutritional density diets.

Replacing glucose with moderate amounts of alpha-cellulose did not stimulate food intake or growth rate in a manner similar to that observed in chicks by Peterson et al. ('54). In fact, increasing the alpha-cellulose contents of the diet from zero to 28% (diets F–C) resulted in a corresponding slight decrease in food consumption and fish growth (table 3).

Although fish growth did not alter appreciably with changing dextrin concentration, the efficiency of protein utilization varied widely among the different diets. As dextrin concentration was increased and alpha-cellulose decreased (diets A–F), the protein efficiency ratio increased from 1.65 to 2.37 (table 3), maximum utilization of protein for growth occurring with 20% of dextrin. When alpha-cellulose was omitted from the diets (diets J, L–O), however, the protein efficiency ratios were considerably reduced as compared with the alpha-cellulose containing diets with identical protein to calorie ratios. This suggests that although addition of alpha-cellulose does not stimulate food consumption and fish growth it does enhance the utilization of protein for growth.

Upon termination of experiment 1, the protein and fat content of the test fish were found to be fairly constant (table 4) among those diets with variations in protein to dextrin levels as long as stomach capacity was not a limiting factor (diets C–F, J, L–O). Using the proximate analysis data for the fish at the start and at the end of this study along with the total fish growth, one can estimate the actual efficiency of protein utilization for protein growth (table 4). This calculation indicates that maximum utilization of protein occurred with the diets containing alpha-cellulose as previously suggested by the protein efficiency ratios. Maximum protein

TABLE 4

Efficiency of carcass protein synthesis, percentage liver weight, liver glycogen content and proximate analysis of fish upon termination of experiment 1

Diet	Gm protein gain/ gm protein fed[1] 0–18 weeks	Liver weight/ body weight × 100[2]	Mg glycogen/ gm liver[3]	Proximate analysis on entire fish[4]			
				H_2O	Protein	Fat	Ash
				%	%	%	%
A	0.226	0.89 ± 0.053[5]	10.4	81.0	72.1	18.7	11.7
B	0.365	0.87 ± 0.0087	21.6	76.4	70.6	21.7	10.4
C	0.336	1.14 ± 0.044	31.2	79.3	68.3	24.0	10.4
D	0.334	1.32 ± 0.057	80.1	79.3	68.1	23.9	10.6
E	0.309	1.35 ± 0.023	99.9	78.9	64.9	26.2	9.7
F	0.319	1.44 ± 0.025	121.3	78.6	65.7	25.7	10.1
G	0.254	1.51 ± 0.093	77.5	77.5	64.3	28.2	9.2
H	0.224	1.14 ± 0.036	11.2	79.4	58.6	34.1	9.5
I	0.249	1.62 ± 0.14	75.6	77.3	58.4	36.6	8.7
J	0.256	1.14 ± 0.066	8.5	77.8	64.1	26.6	10.5
K	0.294	1.21 ± 0.044[6]	5.8[6]	77.1[6]	55.6[6]	35.0[6]	9.8[6]
L	0.270	1.14 ± 0.047	23.1	78.2	65.9	26.6	10.0
M	0.271	1.54 ± 0.055	69.3	78.6	65.8	27.4	9.7
N	0.278	1.46 ± 0.040	65.1	78.3	64.3	28.0	9.9
O	0.291	1.51 ± 0.093	94.5	78.3	64.5	27.7	9.8
Initial	—	—	—	80.0	74.1	19.1	9.1

[1] Estimated from carcass composition and total growth.
[2] Average from 20 fish.
[3] Average from 10 livers, carried out in duplicate.
[4] Average from 50–100 fish.
[5] Standard error of mean.
[6] 14 weeks.

utilization occurred with the 10% dextrin —38% alpha-cellulose diet B. These data again support the observation that addition of alpha-cellulose to the fish diets increases the efficiency of protein utilization.

Increasing amounts of dextrin in the diets resulted in a progressive increase in liver glycogen content and a moderate increase in liver size. The high dextrin diets used in the present study did not result in excess deposition of fat since the carcass fat content remained reasonably constant (23.9 to 28.0%) with diets of high nutritional density (diets C–F, J, L–O).

Attempts to improve fish growth by increasing the caloric content of the diets through addition of fat were quite unsuccessful. Diet H, containing a high concentration of alpha-cellulose and no carbohydrate, was a modification of diet A in which the protein to calorie ratio was decreased by doubling the oil content of the diet. This increase in caloric density, however, did not improve fish growth over that realized with diet A. Calculated efficiencies of carcass protein synthesis (table 4) were identical for both diets. This suggests that the increased calories supplied in the form of corn oil were not available to the fish and consequently did not produce the increased growth observed by addition of a comparable amount of dextrin (diet B).

Diet I was an "isocaloric" modification of diet F in which the corn oil content was doubled and the dextrin concentration reduced by the appropriate amount assuming the alpha-cellulose was not available. Both diets presumably had equivalent protein:calorie ratios, however, doubling the fat content at the expense of dextrin (diet I) markedly decreased fish growth. Moreover, the efficiency of protein utilization was reduced in the high fat diet.

Diet K was a modification of the carbohydrate-free diet J in which the lipid, mineral and vitamin content were doubled. Although this diet was inadvertently fed for only 14 weeks and total fish gain and other values are not strictly comparable, this increase in oil content resulted in decreased fish growth (fig. 1).

Increasing the oil content of the diets which were carbohydrate-free (diets H and K) did not result in an increase in liver glycogen content. In addition, an increase in the lipid content of the diet at the expense of dextrin (diet I) resulted in a significant decrease in liver glycogen. These data support the suggestions that the corn oil supplied in the diet could not be utilized by the fish for production of energy since low liver glycogen stores were observed. Some of the large quantity of oil supplied in these diets was indeed absorbed since proximate analysis of fish after feeding these diets (diets H, I, K) showed a substantial increase in total body fat content.

The test diet commonly used at this laboratory contains 50% of protein (diet G). In contrast, a number of diets which contained only 37.5% of protein showed greater fish growth and considerably higher protein efficiency ratios in these studies.

TABLE 5

Effect of carbohydrate source on rate of growth and food intake of Chinook salmon[1] (exp. 2)

Diet	Initial weight/ fish	Gain/fish 0–14 weeks	Cumulative mortalities 0–14 weeks	Cumulative gain/group[2] 0–14 weeks	Total diet fed/group 0–14 weeks	Total protein fed/group 0–14 weeks	Gm gain/ protein fed 0–14 weeks	Gm weight gain/ cal. fed 0–14 weeks
	gm	*gm*	%	*gm*	*gm*	*gm*		
C	0.60	1.42	12.9[3]	1023[3]	2491[3]	465[3]	2.20	0.272
P	0.57	1.39	15.5	1205	2830	529	2.28	0.282
Q	0.57	1.04	14.8	915	2873	536	1.70	0.211
R	0.56	0.90	9.2	819	2497	466	1.75	0.216
S	0.58	1.75	7.6	1627	3847	720	2.26	0.280
T	0.57	1.70	11.0[3]	1185[3]	2995[3]	559[3]	2.12	0.262
U	0.56	1.05	11.7	947	2766	517	1.83	0.227
V	0.57	1.66	14.4	1436	3093	577	2.49	0.308

[1] 500 fish/trough, in duplicate.
[2] Corrected for mortalities and sample removal.
[3] One trough of fish lost at end of 10th week due to water failure.

In experiment 2, 16 groups of 500 Chinook salmon fingerlings (in duplicate) were fed a series of diets containing a variety of carbohydrates. The diets were modifications of diet C in which dextrin was replaced with fructose, galactose, glucosamine, glucose, maltose, potato starch or sucrose, respectively (table 2). Individual weight gains, mortalities and food consumption of these groups are summarized in table 5 and individual group growth curves plotted in figure 2.

Chinook salmon fingerlings achieved maximum growth with either glucose or maltose as carbohydrate sources (diets S and T) as shown in table 5 and figure 2. When these low molecular weight carbohydrates were replaced with dextrin or potato starch (diets C and U), fish growth was suboptimal.

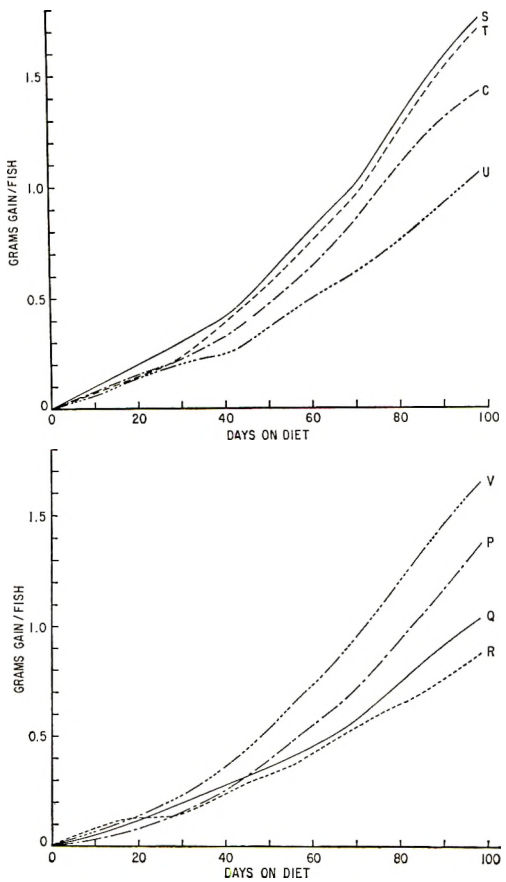

Fig. 2 Growth of Chinook salmon fed different carbohydrates. Weight gains of fish fed dextrin, galactose, glucosamine, glucose, maltose, potato starch and sucrose as the major carbohydrate component of the ration (see table 2).

As the molecular weight of the carbohydrate fed in the diet increased, the total food consumption decreased. In the glucose, maltose, or dextrin diets (diets S, T and C), there was a good correlation of fish growth with the amount of protein consumed and a similarity of protein efficiency ratios. Food consumption was considerably reduced in the case of the potato starch diet (diet U) and, in addition, the fish seemed able to utilize a somewhat smaller percentage of the ingested carbohydrate. This resulted in an increased consumption of dietary protein for energy production as reflected by the decreased protein efficiency ratio (table 5).

Analysis of the carcass gain also showed that the increase of carcass protein per gram of protein consumed was similar for glucose, maltose and dextrin but was reduced for potato starch (table 6). The more precise estimation of actual protein increase gave results that are comparable to those obtained from the more convenient protein efficiency ratio determinations.

Liver weight, liver glycogen and carcass fat content all decreased with increasing carbohydrate molecular weight (table 6) as a result of a decrease in food consumption and a decreased carbohydrate utilization.

When other monosaccharides were substituted for glucose in the experimental diets, fish growth decreased. Fructose (diet P) could support only about 80% of the growth realized with glucose (fig. 2) apparently as a result of a decrease in food intake by the test fish (table 5). The ingested fructose, however, was readily utilized for energy production since the protein efficiency ratio (table 5) and actual efficiency of carcass protein synthesis (table 6) was comparable to that with glucose. The majority of the absorbed fructose was used for energy production rather than stored since liver size, liver glycogen content and carcass fat content were all less than with the glucose-fed fish.

Galactose (diet Q) also caused a decrease in food consumption with Chinook salmon fingerlings. Fish growth was considerably depressed (fig. 2) and the efficiency of protein utilization decreased

TABLE 6

Efficiency of carcass protein synthesis, percentage liver weight, liver glycogen content and proximate analysis of fish at termination of experiment 2

Diet	Gm protein gain/ gm protein fed[1]	Liver weight/ body weight × 100[2]	Mg glycogen/ gm liver[3]	Proximate analysis of entire fish[4]			
				H_2O	Protein	Fat	Ash
				%	%	%	%
C	0.305	1.20 ± 0.052[5]	17.0	80.7	71.8	19.6	11.9
P	0.311	1.09 ± 0.044	29.4	80.5	70.1	20.7	11.5
Q	0.246	1.41 ± 0.074	47.2	80.3	70.3	20.2	11.8
R	0.241	1.23 ± 0.046	8.8	80.2	69.3	20.8	11.2
S	0.311	1.31 ± 0.050	71.0	80.0	68.9	23.3	10.8
T	0.289	1.29 ± 0.053	24.2	80.5	70.0	22.3	11.3
U	0.240	1.14 ± 0.040	23.6	81.1	69.5	21.0	11.8
V	0.343	1.17 ± 0.058	46.4	80.2	69.7	21.5	11.4
Initial	—	—	—	79.8	73.8	19.3	9.2

[1] Estimated from carcass composition and total growth.
[2] Average from 40 fish.
[3] Average from 10 livers, carried out in duplicate.
[4] Average from 50 to 100 fish.
[5] Standard error of mean.

(tables 5 and 6). Although galactose was not converted to carcass fat to an appreciable extent, the amount stored as liver glycogen increased as did the size of the liver. There was no significant increase in the mortalities of galactose-fed fish nor did histological examination of these fish reveal the presence of cataracts such as found in rats fed galactose-rich diets (Mitchell and Dodge, '35).

Glucosamine (diet R) produced the lowest growth of any of the carbohydrates tested in the present study (fig. 2). Not only was total food consumption depressed but there was also evidence of a lowered utilization of glucosamine for energy production and for storage. This was substantiated by a decrease in protein efficiency ratio, a reduction in carcass fat and a marked reduction in liver glycogen content.

Excellent fish growth (fig. 2) was obtained with a sucrose-containing diet (diet V). Sucrose also produced the highest efficiency of protein utilization observed with the various carbohydrate sources tested (tables 5 and 6). Liver size did not increase significantly although some of the ingested carbohydrate was stored as liver glycogen and carcass fat (table 6).

No significant increase in fish mortalities was observed in any of the present studies. In addition, an histologist[4] carried out careful histological examination of all test groups of fish and detected no significant pathology.

DISCUSSION

This study indicates that Chinook salmon fingerlings fed nutritionally adequate diets can tolerate carbohydrate levels as great as 48% for long periods without apparent deleterious effects. These carbohydrate-rich diets produced good fish growth with no increase in mortalities or gross liver pathology; the findings are in agreement with those of McLaren et al. ('47) who carried out somewhat similar studies with trout. They differ, however, from those of Phillips et al. ('48) and the earlier studies of McLaren et al. ('46) who observed high mortalities and enlarged glycogen-rich livers in rainbow trout fed meat or semipurified rations containing large amounts of carbohydrate. With the recent demonstration of normally high amalase levels in the gastrointestinal tracts of fresh water fish (McGeachin and Debnam, '60) along with the results of the present study, however, it appears that such fish can readily absorb and utilize very high carbohydrate levels provided they are supplied with an otherwise nutritionally adequate diet.

There was no indication in the present study that low molecular weight dietary carbohydrates limited food intake via an osmotic effect such as observed in rats by Harper and Spivey ('58). Instead, fish growth and diet consumption decreased

[4] Yasutake, W. T., Western Fish Nutrition Laboratory.

with increasing carbohydrate molecular weight. Addition of small amounts of alpha-cellulose to the test diets increased growth and the efficiency of protein utilization. Although total food consumption remained almost constant, addition of more alpha-cellulose to the test diets decreased total protein and carbohydrate intake. Larger amounts of alpha-cellulose decreased fish growth presumably because the increased dietary bulk did not permit adequate protein or calorie consumption. The stimulatory effect of small alpha-cellulose levels upon growth has also been observed in the chick (Peterson et al., '54) and in guinea pigs (Woolley and Sprince, '45). The mechanism of this growth stimulating effect is unknown but could be the result of: (a) the presence of a trace essential nutrient in the alpha-cellulose; (b) the partial hydrolysis of alpha-cellulose to glucose by intestinal micro-organisms;[5] or (c) the slower passage of alpha-cellulose ration through the gastrointestinal tract and its larger bulk permitting more complete utilization of other dietary constituents.

When fat was exchanged isocalorically for carbohydrate in the diet of Chinook salmon fingerlings, there was a decrease in fish growth and a decreased efficiency of protein utilization. Similarily, increasing the caloric density of carbohydrate-free diets by the addition of fat did not stimulate fish growth and protein utilization. Consumption of diets with increased fat levels was reduced in all cases, perhaps due to a lack of palatability to the test fish. Thomson and Munro ('55) also observed that the exchange of fat for carbohydrate in the diets of rats resulted in decreased protein utilization for growth.

In addition to the adverse effect of fat upon the food consumption of Chinook salmon, these studies indicate that the fat which was ingested and absorbed was not readily utilized for energy production. This follows from the observation of low liver glycogen levels and the decrease or lack of change in protein efficiency ratio following an increase in the dietary fat content.

Immature Chinook salmon were capable of utilizing glucose, maltose, sucrose, dextrose or potato starch although fish growth decreased considerably with increasing carbohydrate molecular weight. Thus, there was no evidence for a lack of gastrointestinal glycosidases as has been observed in immature pigs (Becker et al., '54) or calves (Dollar and Porter, '57). Even though the diet of young salmon normally contains very little carbohydrate, it appears that such fish are well supplied with the enzymes necessary for the breakdown and utilization of polysaccharide (McGeachin and Debnam, '60; Tarr, '58).

Studies by Phillips and co-workers ('48) indicated that brook trout fed beef spleen-mutton tallow diets containing 41% of carbohydrate showed increased mortalities and liver damage when cooked starch, sucrose or dextrin served as the carbohydrate source. Raw starch and cellulose had no ill effect upon fish growth. McLaren et al. ('46) had previously reported that rainbow trout showed growth inhibition and abnormal livers when fed a purified casein-yeast-liver diet containing more than 20% of carbohydrate. Sucrose, lactose, starch and glucose appeared to be equally effective in producing the deleterious effects upon fish growth. Supplementation with riboflavin, pyridoxine and choline did not appreciably improve growth and did not obviate liver damage.

It is well known that the vitamin requirements of experimental animals can vary depending upon the type of carbohydrate supplied in the diet (Mannering et al., '44; Hundley, '49). The growth and health of fish, therefore, could be adversely affected by feeding carbohydrate-rich and nutritionally inadequate diets.

In subsequent studies McLaren et al. ('47) found that skim milk-based diets supported normal rainbow trout growth with carbohydrate levels as high as 45%. In addition, these studies with Chinook salmon have indicated no growth inhibition or liver pathology with 48% of dextrin in the diet. Moreover, when a number of carbohydrate sources were compared using otherwise nutritionally adequate diets, fish growth was noted to vary with the digestibility of the particular carbohydrate. There

[5] Johnson, R. B., and D. A. Peterson 1960 Cellulose digestion by the rat, a nonruminant. Federation Proc., *19:* 321 (abstract).

was no indication of a leveling off in fish growth, excessive mortalities or liver damage with any of the carbohydrates tested.

No significant difference was observed in the growth of Chinook salmon fingerlings fed glucose or sucrose although the fish ingested more food with the glucose diet. Fructose, however, gave only about 80% of the growth obtained with glucose. Winitz et al. ('57) found the growth of rats to be similar with glucose- and sucrose-containing diets, but with a fructose diet rat growth was decreased to 80% of that from glucose, primarily as the result of a decrease in diet consumption.

Apparently no detrimental effect was produced on the fish used in the present study when a galactose-containing diet was fed. Although fish growth was reduced to only 60% of that obtained with glucose, there was no sign of fish pathology and only a slight liver hypertrophy. Reduced growth was not associated with an appreciable decrease in food intake but instead represented a decreased carbohydrate absorption or utilization. In contrast, Landau et al. ('58) observed no difference in the growth of rats fed 25% of glucose or galactose in the diet. In addition, they were not able to demonstrate any alteration in liver enzyme levels produced by feeding galactose which resulted in a decrease in galactose utilization.

It was necessary and challenging to examine the ability of Chinook salmon to metabolize glucosamine since it could conceivably serve as both a source of nonprotein nitrogen as well as carbohydrate. In addition, since chitin is a normal constituent in the diet of fish, the absorption and utilization of glucosamine was entirely possible. The results of this study were equivocal, however. Since fish growth was only about 50% of that observed with glucose, it is apparent that glucosamine does not enhance fish growth; but it is not clear whether glucosamine is completely inert or is utilized to only a small extent. Winitz et al. ('57) noted that glucosamine-containing diets retarded the growth of rats primarily because of a decreased diet intake by the test animals. There was no indication from their studies whether glucosamine was utilized *per se*.

SUMMARY

The effect of different levels of dextrin, alpha-cellulose and fat was studied in the diets of Chinook salmon fingerlings. No appreciable differences in fish growth for diets containing zero to 48% of dextrin and no alpha-cellulose were observed and no deleterious effects of high carbohydrate levels upon fish growth or health were detected. When the alpha-cellulose level of the diet was varied inversely with dextrin concentration, however, fish growth was retarded at very high alpha-cellulose concentrations, presumably as the result of the increased dietary bulk. Small amounts of alpha-cellulose in the diet apparently increased the efficiency of protein utilization. Replacing dextrin isocalorically with corn oil inhibited fish growth and decreased protein synthesis. Since carcass fat content increased markedly, a considerable portion of the dietary fat was absorbed.

In addition, the effect of different carbohydrate sources was examined. When glucose, maltose, dextrin and potato starch were compared, fish growth rate was observed to decrease with increasing carbohydrate molecular weight. Sucrose produced a growth comparable to that with glucose while with fructose fish growth rate decreased 20%. Galactose gave retarded growth but did not result in increased mortalities or demonstrable abnormal pathology. Glucosamine as a carbohydrate source yielded the lowest growth of any of the carbohydrates tested.

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Vitamin B_{12} and the Thyroid in Reproduction of Female Rats[1]

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Olcese et al. ('50), Sure ('51), O'Dell et al. ('51) and Parrott et al. ('60) have demonstrated reproductive failure, including anomalous offspring, in vitamin B_{12}-deficient animals. We have previously reported that in the pregant rat the maternal store of vitamin B_{12} is depleted in favor of that of placenta and fetus, and that human cretins have low serum vitamin B_{12} levels which can be corrected by thyroid therapy (Hellegers et al., '57). Since the lack of thyroid hormone is known to reduce reproductive ability both in man and in experimental animals, it is conceivable that vitamin B_{12} and the thyroid are closely related in the reproductive process. This paper presents exact experimental evidence of the interrelationship between vitamin B_{12} and the thyroid hormone in the reproduction of rats.

EXPERIMENTAL

Animals. Male and femal rats of the McCollum strain from our own colony were used unless otherwise stated. They were 4 to 6 months old, and free from detectable abnormalities. The females had never been bred. In some cases, the thyroidectomized rats were obtained, together with their control animals, from a commercial source.[3]

Breeding. To aid the production of vitamin B_{12} deficiency or inhibition of the vitamin B_{12} action in tissues, the following two agents were used. (a) Inhibitory intrinsic factor (IF)[4] prepared from hog stomach mucosa. Such preparations have been shown to interfere with the absorption of vitamin B_{12} (Williams et al., '57). (b) Vitamin B_{12} antagonist, an anilide of cyanocobalamin.[5]

Determination of vitamin B_{12} activity in plasma and liver. Vitamin B_{12} concentrations in plasma and liver specimens were determined according to the procedure previously described (Yamamoto et al., '57), using Skeggs' medium and *Lactobacillus leichmannii* no. 4797 as the test organism.

RESULTS

Effect of vitamin B_{12} deprivation on reproduction. It is our experience that rats raised and maintained with a vitamin B_{12}-free soybean diet for a long period (three months or longer) lose some reproductive ability. Since the soybean is deficient in other nutrients, such as sulfur-containing amino acids, it can be argued that reproductive failure may not primarily be due to deficiency of vitamin B_{12} alone. In order to minimize the deficiency of dietary protein components, our experimental animals were placed on a good stock diet containing sources of adequate proteins, such as milk. Vitamin B_{12} deficiency was induced by the supplementation of hog intrinsic factor which was shown to decrease vitamin B_{12} absorption, or by the administration of vitamin B_{12} antagonist. Fifty-two young adult female rats were divided into 6 groups (table 1). Five groups were fed our stock diet which has the following components: (in pounds) rolled oats, 18; corn, 20; whole milk powder, 12.5; skim

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[2] Present address: Yamaguchi Medical College, Yamaguchi Prefecture, Ube, Japan.
[3] Thyroidectomized rats obtained from Charles River Breeding Laboratories, North Wilmington, Massachusetts.
[4] Intrinsic factor obtained from Lederle Laboratories, Division of American Cyanamid Company, Pearl River, New York. It was found that the oral administration of this experimental preparation with vitamin B_{12} decreases the gastrointestinal absorption of this vitamin.
[5] Kindly supplied to us by Dr. E. Lester Smith, Glaxo Laboratories, England.

TABLE 1

Relationship between vitamin B_{12} serum levels and reproduction

	Diet and supplement	Body weight	Serum vitamin B_{12}	No. rats mated/no. pregnant	No. rats pregnant/ no. births
		gm	*μg/ml*		
A	Control (stock)	180	0.68	10/10	10/10
B	Stock + inhibitory intrinsic factor[1]	147	0.41	12/3	3/3
C	Stock + inhibitory intrinsic factor + vitamin B_{12}[2]	192	2.0	8/8	8/8
D	Stock + anti-vitamin B_{12}[3]	131	0.71	6/0	0/0
E	Stock + anti-vitamin B_{12} + vitamin B_{12}[2]	188	2.0	6/6	6/6
F	Soybean diet	210	0.40	10/4	4/3

[1] Inhibitory intrinsic factor, 3.5 gm/kg.
[2] Vitamin B_{12} as subcutaneous injection (5 μg three times/week).
[3] Vitamin B_{12}-anilide (50 μg/kg).

milk powder, 12.5; commercial milk protein,[6] 4; NaCl, 0.5; $CaCO_3$, 120; $CuSO_4$, 20; liver powder,[7] 2; ground whole wheat, 30; and ferric citrate, 50 gm. Inhibitory IF was added to the stock diet at the level of 3.5 gm/kg diet for two groups, and vitamin B_{12} antagonist at the level of 50 μg/kg diet for another two groups. One of each of the two groups received in addition 5 μg of vitamin B_{12} by subcutaneous injection three times a week. For comparison, the last group (F) was offered a vitamin B_{12}-free soybean diet. The animals were fed the several diets for 8 weeks and then mated.

The results show that plasma vitamin B_{12} levels of the groups receiving inhibitory IF (B) and soybean diet (F) were considerably lower than that of the control group (A). All of the 10 control rats became pregnant and delivered normally. Fertility in the groups receiving inhibitory IF (B) or soybean diet (F) was low. Only three out of 12 females of group B and 4 out of 10 of group F bore young. None of the 6 rats in group D (vitamin B_{12} antagonist) showed evidence of pregnancy. Administration of vitamin B_{12} to rats treated with inhibitory IF or vitamin B_{12} antagonist (C and E) resulted in fertility and viable young. There were fewer young in the litters of the dams of groups B and F and the incidence of still births in these groups was high. As previously mentioned, these females also had low plasma levels of vitamin B_{12}.

Effect of diet on reproduction and liver vitamin B_{12} concentration of the fetus. Since the preceding experiment demonstrated decreased reproductive capacity of the rats which had lower vitamin B_{12} levels, it was of interest to determine whether the offspring of such animals had correspondingly low tissue vitamin B_{12} concentrations. Accordingly, 48 female rats which had been on the stock diet were randomly divided into three groups. Group A received the soybean diet; group B, the soybean diet supplemented with vitamin B_{12} (1 μg/kg diet), and group C continued to receive the stock diet. Half of the animals in groups A, B and C were mated. At delivery, both the dams and offspring were sacrificed. Vitamin B_{12} concentrations of the livers of the dams and of one young from each litter were determined. These results are tabulated together with the interval between the time of mating and delivery, and number of live young born. Six of the 8 females in group A and all of those in groups B and C delivered young. The average interval between the time of mating and birth of young was considerably greater and the number of live young born was less in group A than in the other two groups (table 2). There was no difference in vitamin B_{12} concentrations in the fetal livers among the three groups and the fetal concentrations were approximately one-half or less than those of the maternal livers.. It is possible that in the fetal liver there is a critical level of vitamin B_{12} below which viable progeny will not develop.

[6] Casal is a commercial preparation of milk protein, Crest Food Company, Ashton, Illinois.
[7] Wilson Company, Chicago.

TABLE 2

Effect of feeding soybean diet on reproduction and vitamin B_{12} content of fetal liver

Diet	No. rats/ no. births	Average interval between mating and delivery (days)	Av. no. live young/ litter	Liver vitamin B_{12} concentration	
				Dam	Young
				$\mu g/gm$	
A Soybean	8/6	33	5.4	71.2±12.9 (81.2±14.7)[1]	36.4±3.5
B Soybean + vitamin B_{12}, 1 μg/kg	8/8	28	8.8	68.1±16.8 (87.1±13.8)	34.0±0.9
C Stock	8/8	26	10.1	87.1±14.6 (99.7±11.8)	37.8±2.9

[1] Liver vitamin B_{12} levels of the control group fed the same diet, but not mated.

TABLE 3

Interrelationship between thyroid activity and vitamin B_{12} in reproduction

Group	No. rats used/ no. pregnant	No. rats pregnant/ births
A Control	6/6	6/6
B Thyroidectomized	12/0	0/0
C Thyroidectomized + vitamin B_{12}[1]	12/5	5/0
D Thyroidectomized + desiccated thyroid (0.05%) U.S.P.	12/1	1/0
E Thyroidectomized + desiccated thyroid (0.01%) U.S.P.	12/4	4/0
F Thyroidectomized + vitamin B_{12}[1] + desiccated thyroid (0.05%) U.S.P.	12/10	10/10
G Thyroidectomized + vitamin B_{12}[1] + desiccated thyroid (0.01%) U.S.P.	12/12	12/0

[1] Five micrograms vitamin B_{12} by injection, three times/week.

Inadequate amounts of vitamin B_{12} in the diet of the female during pregnancy may be reflected in a reduction of vitamin B_{12} in fetal livers, and a drop in live births.

The effect of vitamin B_{12} and thyroid hormone on reproduction in thyroidectomized rats. The absorption of radioactive vitamin B_{12} is markedly reduced in thyroidectomized rats.[8] Such animals also suffered impaired reproduction. It was therefore, of interest to determine the effect of the administration of vitamin B_{12} and/or thyroid on the reproductive ability of thyroidectomized rats. The following experiment was carried out. Of a large number of female rats, 72 were thyroidectomized, and 6 were sham-operated as controls (group A, table 3); all animals were fed the stock diet. The thyroidectomized rats were divided into 6 groups, 12 per group; one group (B) received no treatment; the second group (C) was given by injection vitamin B_{12}, 5 μg three times weekly; the third group (D) received desiccated thyroid (U.S.P.) in the diet, at the level of 0.05% (50 mg/100 gm diet); the 4th group (E), desiccated thyroid (U.S.P.) at the level of 0.01% (10 mg/100 gm diet). The remaining two groups (F and G) received vitamin B_{12} by injection in addition to the two levels of desiccated thyroid. After 4 weeks, all the animals were mated, and the fertility and live birth rates were determined.

As shown in table 3, fertility of thyroidectomized rats (B) was nil, whereas all 6 of the control group (A) became pregnant and delivered normally. Administration of vitamin B_{12} alone (group C) improved fertility in 5 out of 12 rats, but none of these 5 pregnant animals had living young. Administration of desiccated thyroid U.S.P. at the level of 0.05% (group D) or 0.01% (group E) in the diet re-

[8] Okuda, K., S. L. Steelman and B. F. Chow 1956 Absorption of vitamin B_{12} in hyper- and hypothyroid rats. Federation Proc., *15:* 567 (abstract).

TABLE 4

Plasma levels of vitamin B_{12} after thyroidectomy

Group	Months after thyroidectomy	
	1	2
	mμg/ml	
Control	1.03 ± 0.04[1] (5)[2]	0.98 ± 0.06 (4)
Thyroidectomized	0.86 ± 0.05 (5)	0.75 ± 0.07 (4)
P value	< 0.05	< 0.05

[1] Figures are mean ± S.D.
[2] Numbers in parentheses denote number of rats used.

sulted in fertility in one or 4 out of the 12 rats in each group. Administration of both vitamin B_{12} and desiccated thyroid at the level of 0.05% (group F) markedly improved fertility and the birth of live young. Although the rats which received 0.01% of desiccated thyroid in addition to the injections of vitamin B_{12} all became pregnant, none produced viable young. These data demonstrate the interdependence of the supplies of vitamin B_{12} and thyroid extracts.

Males used as breeders for groups B through E in which reduced fertility was observed were later tested for potency. Each was mated with three females. All 15 males appeared to be potent as evidenced by the fact that 43 out of 45 females became pregnant and delivered living young.

These results demonstrate that thyroidectomy reduces reproduction in female rats and the infertility thus produced can be only partially improved by the administration of vitamin B_{12} or desiccated thyroid. Administration of both, however, was more effective in improving reproduction.

Plasma vitamin B_{12} levels after thyroidectomy. Since infertility in thyroidectomized rats was partially corrected by the administration of vitamin B_{12}, and since the plasma level of the vitamin is a good index of the amount available in the body, it was of interest to determine this level in thyroidectomized animals. For this purpose, 10 two-month-old male rats were used. One-half of them were thyroidectomized and the other half served as controls. All animals were fed the stock diet throughout the experimental period. Two months later, the plasma vitamin B_{12} levels of both groups were determined. The results (table 4) show that plasma concentration of vitamin B_{12} decreased significantly after thyroidectomy.

DISCUSSION

Removal of the thyroid gland reduced fertility in female rats. This was partially corrected by the injection of vitamin B_{12}. This suggests that some degree of vitamin B_{12} deficiency develops in thyroidectomized animals. The demonstration of lowered plasma levels of vitamin B_{12} in thyroidectomized rats supports this premise. Since thyroid performs a number of metabolic functions, it is unlikely that reduction in vitamin B_{12} absorption after thyroidectomy is the sole cause of reduction of fertility in athyroidism. Infertility after thyroidectomy was partially but not completely corrected by the administration of desiccated thyroid. Satisfactory reproduction in thyroidectomized rats was obtained only with the combination of vitamin B_{12} and desiccated thyroid at 0.05 % level of the thyroid in the diet. The same combination failed to bring about a normal birth rate when the level of desiccated thyroid was lowered to 0.01%. It is conceivable that vitamin B_{12} and the thyroid hormone are closely related in the reproduction process, and are interdependent in certain biochemical processes during the fetal growth.

SUMMARY

Studies were conducted on female rats on the effects of vitamin B_{12} deprivation and thyroidectomy. The following results were obtained. (a) Female rats, when depleted of vitamin B_{12} either by feeding a vitamin B_{12}-free soybean diet, or by administration of inhibitory intrinsic factor, or by use of vitamin B_{12} antagonist in a complete diet, showed impairment of reproduction. Administration by injection of

vitamin B_{12} corrected both fertility and live birth rate in such animals. (b) Thyroidectomized females did not become pregnant when bred to males of established potency. The infertility due to thyroidectomy was partially corrected by the administration of either vitamin B_{12} or desiccated thyroid. Administration of both vitamin B_{12} and desiccated thyroid fed at a level of 0.05% greatly improved reproduction in thyroidectomized rats. The probable interrelationship between vitamin B_{12} and thyroid hormone in the reproduction of rats has been discussed.

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Effects of 3:5:3′-Triiodo-D-Thyronine on Serum Vitamin A and Carotenoids in Hypothyroidism[1]

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The role of thyroid hormone in the conversion of β-carotene to vitamin A remains unsettled (Lowe et al., '56; Moore, '57). Moore ('57) in a recent summary concluded that changes in serum vitamin A associated with changes in thyroid function are due to "drifts with the metabolic tide." Although this statement has little meaning, it implies that changes in serum vitamin A follow changes in oxygen consumption. β-Carotene, a precursor of vitamin A, is transported in the lipoprotein fraction of serum along with cholesterol. A newly synthesized D-isomer of triiodothyronine (D-T_3) causes in euthyroid subjects a more pronounced effect on serum cholesterol than on oxygen consumption. This compound therefore should be of value in indicating whether changes in serum carotenoids and vitamin A are related more closely to changes in oxygen consumption or to changes in serum cholesterol.

In this paper the effects of D-triiodothyronine on serum cholesterol, carotenoids, vitamin A and oxygen consumption in a group of patients with thyroid deficiency are reported with a note added regarding the effect of D-triiodothyronine on liver storage of vitamin A in rats.

METHODS

Five patients with myxedema induced by radioiodine because of severe heart disease were studied. At the time of study all patients showed evidence of decreased thyroid function as measured by oxygen consumption, serum protein bound iodine and radioactive iodine uptake. Two separate study periods were included. A preliminary study was done in the spring of 1960 and a more detailed one was carried out three months later. Two healthy subjects (WE and JM) served as controls during the second study period. Serum cholesterol was measured according to the method of Pearson et al. ('52). Serum carotenoids and vitamin A were estimated by the method of Roels and Trout ('59). Oxygen consumption was estimated by collecting expired air samples in a Douglas bag, the volume measured in a Tissot spirometer and the percentage of oxygen estimated by either the Scholander method or by the Pauling oxygen analyzer. The oxygen consumed per minute corrected for body surface area is expressed as oxygen consumption index.

For the experimental animal studies, adult male Sprague-Dawley rats were used and the thyroids were surgically removed. The diet consisted of commercial rat pellets.[3] After an average of 84 days the animals were pair-fed a synthetic diet containing no vitamin A, having the following percentage composition: casein, 24; sucrose, 62; commercial hydrogenated oil,[4] 10; and salt mix W, 4. A vitamin mixture (mg/100 gm) was added to the basic ration as follows: thiamine·HCl, 0.5; riboflavin, 0.5; pyridoxine·HCl, 1.0; Ca pantothenate, 1.0; inositol, 1.0; niacin, 0.2; *p*-aminobenzoic acid 0.2, choline chloride, 100.0; and biotin 0.002. After supplying the diet for 10 days, the distal portion of the left lobe of the liver was removed under ether anesthesia and its vitamin A content determined. Five days later, one half of the animals were started on 45 μg of D-T_3 per kg of body weight per day. This dose was chosen because earlier work in-

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[3] Purina, Ralston Purina Company, St. Louis.

[4] Crisco, Procter and Gamble, Cincinnati.

dicated that this amount of D-T_3 did not increase oxygen consumption of euthyroid rats but was effective in preventing thiouracil-induced goiter. The remaining half of the animals received daily injections of saline. For 4 days prior to sacrifice, all animals were given by mouth 515 μg per day of β-carotene in a cottonseed oil[5] solution. Total fecal collections were made throughout the course of the carotene feeding and were analyzed for β-carotene content. The animals were then killed and the liver analyzed for vitamin A and carotene.

For the oxygen consumption studies the authors are indebted to Doctor Janet Lemley-Stone who measured Q_{O_2} of diaphragm slices in the Warburg apparatus, 37°C, 100% oxygen atmosphere in Krebs-Ringer phosphate buffer.

RESULTS

The effects of D-triiodothyronine on the serum constituents and oxygen consumption in the patients during the preliminary period of treatment are summarized in table 1. In each instance serum cholesterol decreased with administration of the drug. The cholesterol values before and during treatment are the average of values obtained. In this preliminary study there was a suggestion of a similar decrease in serum carotenoids although this was prominent only in patient LM. Serum vitamin A increased in one patient but in three, little change occurred. Oxygen consumption increased in all patients with administration of D-triiodothyronine; this change was most prominent in patients JE and RH.

Changes in serum cholesterol, carotenoids and oxygen consumption were not accompanied by consistent changes in vitamin A. Hence the drug was withdrawn to repeat the study after the patients had returned to their pretreatment state. After three months, D-triiodothyronine was again given to 4 of these patients and to two control subjects. All received 0.4 mg daily for 21 days except patient CB who received the drug for 17 days. The results are summarized in table 2.

In the second studies the serum cholesterol decreased in all subjects with administration of the drug. A decrease in serum carotenoids followed administration of D-triiodothyronine. The largest fall in carotenoids occurred in the patient with the highest serum cholesterol who also had a large increase in oxygen consumption with administration of the thyroid analogue. Patient JE had a similar increase in oxygen consumption but little change in serum carotenoids. His pretreatment serum carotenoid value was the lowest of the entire group.

The response of serum vitamin A to the administration of D-triiodothyronine was

[5] Wesson Oil, Wesson Oil Company, New Orleans, Louisiana.

TABLE 1

Effects of D-T_3 during the first study

		Cholesterol	Carotenoids	Vitamin A	O_2 consumption index
		mg/100 ml	*μg/100 ml*	*μg/100 ml*	*ml O_2/M^2 BSA*
LM	Pre-treatment	278	171	41	97
	0.4 mg daily, 5 days	208	113	44	108
	Post-treatment	258	—	—	86
JC	Pre-treatment	297	215	59	88
	0.4 μg daily, 51 days	261	211	63	104
	Post-treatment	254	—	—	123
JE	Pre-treatment	346	172	58	76
	0.4 mg daily, 6 days	314	162	70	94
CB	Pre-treatment	358	114	48	82
	2.9 mg over 23 days	323	91	47	85
	Post-treatment	382	76	54	72
RH	Pre-treatment	350	—	—	45
	0.5 mg daily, 5 days	217	—	—	88

TABLE 2

Effects of D-T_3 during the second study

		Cholesterol	Carotenoids	Vitamin A	O_2 consumption index
		mg/100 ml	*μg/100 ml*	*μg/100 ml*	*ml O_2/M^2 BSA*
LM	Pre-treatment	256	139	39	111
	0.4 mg daily, 21 days	198	117	28	123
	Post-treatment	228	139	34	120
JC	Pre-treatment	301	256	56	117
	0.4 mg daily, 21 days	265	227	61	119
	Post-treatment	250	223	63	117
JE	Pre-treatment	323	106	50	72
	0.4 mg daily, 21 days	207	101	45	112
	Post-treatment	226	95	27	70
CB	Pre-treatment	473	229	49	80
	0.4 mg daily, 17 days	382	160	49	107
	Post-treatment	418	188	63	105
WE	Pre-treatment	231	122	58	116
	0.4 mg daily, 21 days	209	99	57	128
	Post-treatment	239	104	64	—
JM	Pre-treatment	304	176	62	—
	0.4 mg daily, 17 days	237	166	65	—
	Post-treatment	254	160	60	—

not consistent. In patient LM serum vitamin A decreased; in patient JC serum vitamin A increased as it had in the preliminary study. Patient JE showed an increase in serum vitamin A with the drug in the first study but a decrease during the second study.

Oxygen consumption increased with administration of D-T_3 and the increase was more readily demonstrable when the initial value was low.

Upon withdrawal of the drug, serum cholesterol increased promptly in all patients except JC. Changes in serum carotenoids following discontinuation of the drug did not uniformly parallel changes in serum cholesterol. No consistent change was observed in serum vitamin A following cessation of D-triiodothyronine.

Because of the inconsistent response of serum vitamin A, it is not possible to define to what extent the D-triiodothyronine alters either conversion of carotenoids to vitamin A or of vitamin A transport.

Since reciprocal changes in serum vitamin A and carotenoids did not occur with the administration of D-T_3, the effect of D-triiodothyronine on the liver storage of vitamin A in rats was investigated. The results of this study are summarized in table 3. No difference was observed in the average liver weight of the control and experimental animals. The pretreatment total liver vitamin A of 2.7 to 3.9 mg was estimated by using biopsy concentration and total liver weight obtained at necropsy. Liver vitamin A increased in all animals after feeding β-carotene. The post-treatment values were 3.4 to 4.5 mg per whole liver. The increase in vitamin A ranged from 13 to 26%. The percentage increase in liver vitamin A correlated with initial vitamin A content of the liver. A negative correlation (− 0.82) between initial vitamin A content of the liver prior to feeding carotene and the observed percentage increase in vitamin A was found. Two of the animals receiving D-T_3 showed a larger total increase in vitamin A and two a smaller increase when compared with their pair-fed controls not receiving D-triiodothyronine. No consistent changes in vitamin A were noted in those animals receiving the D-triiodothyronine compared with pair-fed controls. It is suggested that administration of D-T_3 at this dose level does not influence liver storage of vitamin A in adult hypothyroid rats.

Approximately ¼th of the administered carotene was recovered in the feces. The

TABLE 3

Effects of D-T_3 on liver storage of vitamin A and fecal excretion of β-carotene in rats

Subject	Vitamin A conc.		Vitamin A content in liver		Carotene excreted	Serum	
	Biopsy	Postmortem	Biopsy	Postmortem		Vitamin A	Carotenoids
	μg/gm		*mg*	*mg*	*μg*	*μg/100 ml*	
Control	276	341	3.4	4.0(29.1)[1]	420(20.3)[2]	60	0
D-T_3	237	329	2.8	3.7(24.3)	502(24.4)	24	0
Control	358	428	3.5	3.9(19.4)	472(22.9)	18	0
D-T_3	249	352	2.7	3.4(34.0)	478(23.2)	28	0
Control	332	435	3.3	4.0(34.0)	515(25.0)	29	0
D-T_3	384	486	3.6	4.2(29.1)	399(19.4)	17	0
Control	377	481	3.5	4.2(34.0)	442(21.5)	29	0
D-T_3	372	454	3.9	4.5(29.1)	266(12.9)	41	0

[1] Percentage of administered carotene recovered in liver.
[2] Percentage of administered carotene recovered unchanged in feces.

absorption spectrum of the substance recovered from the feces was identical with that of the carotene administered. A yellow pigment was demonstrated in liver extract concentrates; however, analysis of these concentrates revealed no substance giving the absorption spectrum of β-carotene. The absorption spectrum was of a nonspecific pattern decreasing in intensity from 380 to 520 mμ.

DISCUSSION

The unsolved relationship between thyroid hormones and metabolism of β-carotene and vitamin A prompted these studies. The availability of D-T_3, said to have more effect on lipid metabolism than on oxygen consumption, presented an opportunity to investigate the effect of this substance on serum carotenoids and vitamin A in patients deficient in thyroid hormone. It was felt by using this drug that increased conversion of carotenoids to vitamin A without increase in body metabolism might be detected. Somewhat unexpectedly, increased oxygen consumption was noted in these patients receiving this small dose of D-T_3. Although changes in carotenoids and cholesterol were produced in a consistent manner, the response of serum vitamin A was unpredictable. Reciprocal changes did not always occur in serum vitamin A with acute changes in serum carotenoids. In other words, enhanced conversion of carotene to vitamin A, as estimated by serum studies, was not observed with administration of D-T_3. Since only serum was sampled in the human studies, it is not possible to state what effect the thyroid analogue may have had on absorption or storage of vitamin A. It has been emphasized by Caster and Mickelsen ('55) that the serum level of vitamin A does not necessarily reflect liver concentration. For this reason it cannot be stated unequivocally that this thyroid analogue is without effect on the conversion of carotene to vitamin A.

The decrease observed in cholesterol was greater than that observed in serum carotenoids. Whether this fall in serum carotenoids is related to an increase in body metabolism or to a specific effect of D-T_3 on the serum lipoproteins remains unknown.

In the rat studies no difference was observed in the liver storage of vitamin A in animals receiving D-T_3 and those receiving saline. All animals increased liver vitamin A stores following administration of β-carotene. Since the rats were adults they had large vitamin A stores in the liver; hence, subtle changes in liver storage would be difficult to detect. It has been suggested that vitamin A reserves are depleted by thyroid hormone as oxygen consumption increases. Oxygen consumption was higher in the diaphragm of the animals given D-T_3 than in that of the controls. It is impossible to state to what extent this increase in metabolism may have influenced the vitamin A content of the liver. No statistically significant difference in the serum vitamin A was found

between the control and experimental animals. Although it has been reported that thyroid hormone promotes absorption of carotene, our studies revealed little difference in absorption of carotene between the control animals and those given this particular thyroid analogue.

SUMMARY

D-Triiodothyronine lowered serum carotenoids and cholesterol but did not change serum vitamin A consistently. Increased oxygen consumption occurred when D-T_3 was given. No evidence was obtained that D-T_3 enhanced conversion of β-carotene to vitamin A.

The study with the thyroidectomized rats suggests that D-triiodothyronine did not increase the amount of liver vitamin A formed from carotene nor alter appreciably the excretion of carotene.

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Observations on the Requirements of Young Chicks for Dietary Fat [1,2]

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The literature on the dietary need of chicks for fat is limited and clouded with contradictory reports. Reiser and Couch ('49) reported that chicks fed a purified, fat-free diet grew at a definitely slower rate than those receiving the same ration supplemented with 4% of cottonseed oil.[4] Reiser ('50a) also reported poor growth as well as high mortality when chicks were fed a purified, fat-free diet. Dam et al. ('59) suggested that the results of Reiser ('50a) may be open to question due to possible thiamin deficiency caused by destruction of that vitamin by sulfite in the "alpha protein" used. Since improved growth and lower mortality resulted when 4% cottonseed oil was added to the fat-free diet, however, it seems improbable that the poorer growth and high mortality could be attributed solely to this vitamin deficiency. Carver and Johnson ('53) and Davis and Upp ('41) also reported poorer growth with fat-free diets.

On the other hand, Russell and associates ('40) reported no detrimental effect on growth to 14 weeks of age when chicks were fed an ether-extracted natural-type ration. They had observed depressed growth, however, in a preliminary experiment which subsequently led to the work reported. Bieri et al. ('56) observed that chicks grew normally for 6 to 8 weeks when raised with a purified diet containing 0.046% of fat. Briggs and Spivey Fox ('55) questioned the need of chicks for dietary fat, yet presented data highly suggestive of a fat requirement. They attributed the difference in growth to poor palatability and loss of unstable nutrients, or to loss of unstable nutrients alone.

In the course of experiments designed to study the effect of dietary fat on cholesterol metabolism, we have made observations which may help explain the conflicting reports on the dietary requirements of chicks for fat.

EXPERIMENTAL

Day-old male chicks from the University of Hawaii strain of New Hampshires were used in all experiments. The dams of these chicks were housed in wire-floor community pens and received a stock University of Hawaii all-mash laying ration. All chicks were brooded in thermostatically controlled battery brooders with raised wire floors; feed, water and lights were provided continuously. Individual body weights were obtained weekly in three separate experiments.

The basal diet used in all three experiments was a modification of the one described by Briggs et al. ('52). The composition of the experimental diets is shown in table 1. The diethyl ether extractable content of the mixed "fat-free" diet was found to be 0.05%. The diet contained, by calculation, 0.07% of fat-soluble vitamins A and E, although it is probable that the gelatin-coated vitamin A was incompletely dissolved by the prolonged ether extraction. The various rations were calculated to be isocaloric, part of the sucrose being replaced by ground silica in those diets that contained fat. Rations were mixed as needed in lots of 5 or 10 kg and sufficient feed to last one or two days was

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[2] The work reported in this paper was supported in part by a grant from the Hawaii Heart Association, Honolulu, Hawaii.
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[4] Wesson Oil, Wesson Oil Company, New Orleans, Louisiana.

TABLE 1

Composition of experimental diets

	Basal ration	3% Corn oil	15% Corn oil
	gm/kg	*gm/kg*	*gm/kg*
Casein	200	200	200
Gelatin	80	80	80
Cellulose[1]	20	20	20
Sucrose	585	515	245
Ground silica	—	40	190
Fat	—	30	150
DL-Methionine	3	3	3
Choline chloride	2	2	2
Mineral mix[2]	60	60	60
Vitamin mix[3]	50	50	50

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[2] Supplied the following in gm/kg of diet: $CaCO_3$, 15; K_2HPO_4, 9; Na_2HPO_4, 7.2; $Ca_3(PO_4)_2$, 14; NaCl, 8.8; $MgSO_4 \cdot 7H_2O$, 5; ferric citrate (16.7% Fe), 0.4; $MnSO_4 \cdot 4H_2O$, 0.42; KI, 0.04; $ZnCO_3$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.02; Na molybdate, 0.10.

[3] Supplied the following in mg/kg of diet: thiamine, 8; riboflavin, 8; Ca pantothenate, 20; niacin, 100; pyridoxine, 8; D-biotin, 0.3; folic acid, 3; vitamin B_{12}, 0.02; vitamin D_3, 0.02; α-tocopheryl acetate, 100; 2 methyl-1,4-naphthoquinone, 1; vitamin A, gelatin coated (500 I.U./mg), 200; glucose, 49,551.66.

placed in the feed troughs at one time. The balance of the mixed rations was stored in a refrigerator until the troughs were empty.

In experiment 1, 50 one-day-old chicks were fed the "fat-free" basal diet for 6 weeks. A control group of 10 chicks was fed the basal diet supplemented with 3% of corn oil.

In experiment 2, 60 one-day-old chicks were divided at random into 6 equal groups. One group served as the control and received the 3% corn oil diet from the first day. The remaining 5 groups received the "fat-free" diet for one week only, and were then fed the experimental rations containing linoleate[5] or fats as shown in table 2. One of these groups continued to be fed the "fat-free" basal diet and served as the negative control group.

Although no postmortem examinations had been planned in experiments 1 and 2,

[5] The level of linoleate used was calculated to be 0.33% on the basis of 0.5% crude (65%) methyl linoleate. The iodine number of a sample of this material was measured at the end of the experiment and found to be 141, indicating some auto-oxidation.

TABLE 2

Mean weekly body weights, feed consumption, and mortality of New Hampshire male chicks fed "fat-free" basal, linoleate- or fat-supplemented diets (experiment 2)

Treatment	Week						Feed consumed[1]	Mortality[2]
	1	2	3	4	5	6		
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*%*
"Fat-free" basal	67	116	201	276	393	517	952	78
"Fat-free" basal + 0.33% linoleate[3]	69[4]	115	200	306	435[5]	607[5]	1001	20
"Fat-free" basal + 3% corn oil	68	127	216	335[6]	467[6]	580[5]	1049	30
"Fat-free" basal + 15% corn oil	68[4]	137[5]	239[5]	361[6]	532[6]	640[5]	1189	18
"Fat-free" basal + 15% MFB[7]	70[4]	146[6]	253[6]	368[6]	496[6]	639[6]	1322	22
"Fat-free" basal + 15% butterfat	69[4]	137[5]	237[6]	324[5]	468[6]	637[6]	1282	20

[1] Mean feed consumed per bird from one to 6 weeks.

[2] Mortality observations were made over a 23-week period and include both dead and dying animals.

[3] Derived from 0.5% crude (65%) methyl linoleate.

[4] These groups received "fat-free" basal for first week.

[5] Significantly greater than corresponding "fat-free" basal, $P = < 0.05$.

[6] Significantly greater than corresponding "fat-free" basal, $P = < 0.01$.

[7] Brand of hydrogenated vegetable oil.

some observations of the occurrence of aspergillosis were made when the animals were sacrificed. Therefore, experiment 3 was designed in an attempt to control this infection with antibiotics. Twelve groups, consisting of 10 chicks each, were used in this experiment, triplicate groups being assigned to each of 4 treatments. In addition to the "fat-free" and 3% corn oil groups, two antibiotic treatments were included. Since fungicidin[6] has been shown by Yacowitz et al. ('59) to be effective aginst certain fungi, it was used as one of the antibiotic treatments (550,000 units per kg of "fat-free" diet). The second antibiotic treatment consisted of a high level of procaine penicillin (0.66 gm of Nopcaine[7] per kg of the "fat-free" basal ration). All surviving chicks in experiment 3 were sacrificed at 14 weeks of age and the left lungs were removed for laboratory diagnosis of aspergillosis.

Vitamin A determinations were made on the livers from some birds in experiments 2 and 3 after the chicks had received the experimental diets for 66, 98, or 112 days. Livers from several chicks of the same age and sex which had received a standard corn-soybean oilmeal ration were also assayed for comparative purposes.

Growth data from experiments 1, 2, and 3 were analyzed statistically by means of the one-tailed "t" test (Cochran and Cox, '50), and the data from experiment 3 were also subjected to the analysis of variance (Snedecor, '56); differences were tested further for significance by means of a multiple range test (Duncan, '55).

RESULTS AND DISCUSSION

Supplementation of the "fat-free" basal diet with 3% of corn oil resulted in a significant increase in growth in all three experiments. The inclusion of linoleate in a "fat-free" diet also resulted in a significant growth response. These results confirm the findings of Davis and Upp ('41), Reiser and Couch ('49), Reiser ('50a), and Carver and Johnson ('53). These workers reported that the growth of chicks fed a "fat-free" diet was improved when fat or fatty acids were included in the ration.

The growth data of experiment 1, presented in table 3, show that a significant growth response was obtained as early as two weeks of age. In experiment 2 (table 2), a significant growth response at two weeks of age resulted when 15% of corn oil, MFB,[8] or butterfat was added to the basal "fat-free" diet. In this experiment, however, although the group receiving the 3% corn oil-supplemented diet grew better than the "fat-free" control group at two weeks, the difference in gain was not statistically significant until the 4th week. At three weeks of age, the growth of the chicks receiving 3% of corn oil in experiment 3 (table 4) was significantly greater than that of the chicks fed the "fat-free" basal. These data suggest that the requirement of the growing chick for dietary fat becomes more critical starting at about two weeks of age. Since Reiser ('50b) presented data which suggested that chicks are unable to synthesize linoleic or linolenic acids to any extent, these results may indicate depletion of the yolk fat in chicks in the first few weeks after hatching.

The 6-week body weights of the chicks receiving 15% of the various fats (table

[6] Mycostatin, courtesy of E. R. Squibb and Sons, New Brunswick, New Jersey.
[7] Courtesy of NOPCo Chemical Company, Harrison, New Jersey.
[8] Brand of hydrogenated vegetable oil.

TABLE 3

Mean weekly body weights of New Hampshire male chicks fed the "fat-free" basal diet with and without 3% of corn oil (experiment 1)

Treatment	Week					
	1	2	3	4	5	6
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
"Fat-free" basal	66	108	167	264	339	430
"Fat-free" basal + 3% corn oil[1]	69	132[2]	239[2]	350[2]	482[2]	593[2]

[1] Mazola, Corn Products Refining Company, New York.
[2] Significantly greater than corresponding "fat-free" basal, $P = < 0.01$.

TABLE 4

Mean weekly body weights, feed consumption, and mortality of New Hampshire male chicks fed a "fat-free" basal, and supplemented with 3% of corn oil or antibiotics (experiment 3)

Treatment	Week						Feed consumed[1]	Mortality[2]
	1	2	3	4	5	6		
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	%
"Fat-free" basal	76	131	215	326	455	602	1102	47
"Fat-free" basal + fungicidin[3]	72	127	208	324	458	589	1078	53
"Fat-free" basal + penicillin[4]	85[5,6]	158[5,6]	242[5]	351	484	643	1119	37
"Fat-free" basal + 3% corn oil	76	138	243[5]	376[5]	544[5]	710[5]	1214	10

[1] Mean feed consumed per bird over 6-week period.
[2] Mortality observations were made over a 13-week period.
[3] 0.45% Mycostatin, which contained 56,000,000 units per pound.
[4] 0.066% Nopcaine, which contained 200 gm of procaine penicillin per pound.
[5] Significantly greater than "fat-free" basal group, $P = < 0.01$ (t test).
[6] Also significantly greater than all other treatments (analysis of variance).

2) were very uniform, suggesting that the type of fat is relatively unimportant with respect to the early growth effect obtained from the inclusion of fats in the diet. The increased growth response of the 15% corn oil group over the 3% corn oil group tends to substantiate the finding of Dam et al. ('59) who reported highly significant growth increases when the vegetable oil content of purified diets was increased from 3 to 5 to 10%.

The feed consumption for experiments 2 and 3 are shown in tables 2 and 4, respectively. In both experiments the 3% corn oil groups consumed about 10% more feed than the "fat-free" groups. This might indicate that the fat content of the diets may have increased nutrient intake which, in turn, would increase growth. Nutrient intake, however, is not necessarily correlated with growth. The groups receiving 15% of MFB and butterfat consumed more feed than the group receiving 15% of corn oil but did not make any better growth. On the other hand, the chicks receiving the linoleate consumed less feed and made better growth than those receiving 3% of corn oil.

Bieri et al. ('56) suggested that the poor growth and high mortality resulting from feeding chicks a "fat-free" purified diet, as reported by Reiser ('50a), may have been the result of a vitamin A deficiency due either to the instability or poor absorption of this vitamin. Therefore, in the experiments reported here, a very high level of gelatin-coated vitamin A was included in all rations and, in addition, liver vitamin A analyses were made. The livers were taken from birds which had been fed the experimental diets continuously for periods of 66, 98, or 112 days. It would be expected that if a vitamin A deficiency resulted from the continuous feeding of the purified "fat-free" diet, the deficiency would become progressively more severe. It is obvious from the data shown in table 5 that no vitamin A deficiency had occurred; in fact, the liver vitamin A values for all treatment groups tested were much higher than those from chicks receiving a natural corn-soybean oilmeal type ration. Furthermore, rather than decreasing, the liver vitamin A progressively increased with age. These data show that large amounts of vitamin A were absorbed by the chicks from the "fat-free" diet.

Although fungicidin was without effect on the growth of chicks receiving the "fat-free" diet, a highly significant growth response was obtained when penicillin was included in the ration (table 4). For the first two weeks this growth response was significantly greater than that obtained from the addition of 3% of corn oil. An analysis of variance of the 6-week growth data showed that the 3% corn oil group made greater gains than any other group in

TABLE 5

Mean liver vitamin A of chicks fed purified diets with and without added fat, and a natural corn-soybean oilmeal-type diet

Treatment	Vitamin A[1] in feed	Age at time of analysis (days)		
		Experiment 2		Experiment 3
		66	112	98
	I.U./100 gm	*I.U./gm*	*I.U./gm*	*I.U./gm*
"Fat-free" basal	10,000	1663(2)[2]	5563(2)	4556(10)
"Fat-free" basal + 3% corn oil	10,000	—	—	3505(10)
"Fat-free" basal + 15% corn oil	10,000	2800(1)	6100(1)	—
Corn-soy diet	550	38(1)	105(1)	184(2)

[1] Calculated.

[2] Figures in parentheses represent number of livers analyzed.

experiment 3, and that the penicillin-fed group gained significantly faster than either the "fat-free" basal or fungicidin-fed groups.

There was no appreciable mortality in any of the groups at 6 weeks of age. Since the chicks in experiments 2 and 3 were fed the experimental diets continuously for 23 and 13 weeks, respectively, these data are presented in the last columns of tables 2 and 4. In both experiments the mortality in the "fat-free" groups was appreciably higher than in the groups receiving fat or fatty acids. The mortality in the fungicidin group was as high as in the "fat-free" control group, although mortality in the penicillin group was somewhat lower.

The laboratory examination of the lungs from the surviving birds in experiment 3 showed that all specimens contained *Aspergillus*. This finding, together with the postmortem observations in experiment 2, suggests that *Aspergillus* infection may have been partly responsible for the poorer growth and mortality observed in the "fat-free" groups. If this were the case, then the presence of *Aspergillus* in the fat-fed as well as the "fat-free" groups further suggests that the fat-fed birds were more resistant to this infection. While *Aspergillus* was the only disease organism demonstrated in this work, it is very likely that other microorganisms may also have contributed to the poorer growth of the "fat-free" chicks. The improved growth of the "fat-free" plus penicillin groups would tend to support this statement.

Other workers have observed growth differences in chicks attributable to subclinical infections in certain environments. Coates et al. ('51, '52), studying the effects of new and long established quarters on the response of chicks to antibiotics, found that chicks reared in new quarters failed to show a growth response to penicillin. They suggested that the chicks in the long established quarters had an infection which, although not grossly apparent, responded to penicillin. Bird et al. ('52), Hill et al. ('53) and Lillie et al. ('53) presented further evidence in support of this hypothesis.

Research conducted with another species indicates that one of the primary roles of dietary fat is related to disease resistance. Hansen and associates ('48) observed over a 4-year period that young dogs fed low-fat diets showed an apparent lowered resistance to infection. Predominant observations were emaciation, pneumonia, and skin and ear infections. Worden ('58) also reported that increased susceptibility to infection was a constant feature observed in dogs fed low-fat diets.

If a similar mechanism is operative in the chick, then the degree of contamination in the environment might be an important factor in determining whether a growth response would be obtained from the inclusion of dietary fat.

SUMMARY

In three experiments conducted with New Hampshire chicks, highly significant growth increases were obtained as early

as two weeks by the addition of 3% of corn oil to the "fat-free" (0.05% fat) basal diet. An additional response was obtained by the supplementation of the basal diet with 15% of corn oil, a hydrogenated vegetable oil, or butterfat. Responses to the different fats were of equal magnitude. Supplementation of the "fat-free" diet with 0.33% of methyl linoleate also resulted in significantly increased growth.

The growth response obtained from penicillin was significantly greater than that obtained with 3% of corn oil at two weeks of age, but was significantly less at 6 weeks. The 6-week body weights of the groups receiving the 3% corn oil or the penicillin treatment were significantly greater than those of the "fat-free" basal group. Fungicidin in the "fat-free" diet was without apparent effect.

Liver vitamin A analyses showed clearly that the poorer growth of the "fat-free" groups was not due to a vitamin A deficiency, and further indicated that gelatin-coated vitamin A was absorbed in large amounts from "fat-free" diets by the chick.

Postmortem observations showed symptoms of aspergillosis. This infection was more severe in the "fat-free" groups.

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Essential Fatty Acid Nutrition in Swine

I. LINOLEATE REQUIREMENT ESTIMATED FROM TRIENE: TETRAENE RATIO OF TISSUE LIPIDS[1]

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Preliminary studies on essential fatty acid (EFA) deficiency of swine by Hill et al. ('57) did not reveal dermal lesions in this species. Analysis of lipids from several swine tissues, however, showed the biochemical lesion characteristic of essential fatty acid deficiency in other species, that is, diminished dienoic and tetraenoic fatty acids and elevated trienoic fatty acids. This biochemical lesion has been reported for rats (Rieckehoff et al., '49), chicks (Bieri et al., '57) and dogs (Wiese and Hansen, '51). Recently Peifer and Holman ('59) have shown that the EFA requirement of the rat increases when the saturated fat in a diet increases, indicating that the EFA requirement may be dependent in part upon the total fat calories of the diet. More recently, Holman ('60) has related the polyunsaturated acid content of heart tissue and blood lipids of the rat to the dietary linoleic acid content, expressed as percentage of calories, and has shown that a ratio of triene/tetraene in blood or heart lipids of more than approximately 0.4 indicates EFA deficiency, and a ratio of 0.2 or less that the minimum requirement of linoleate has been met. The purpose of the present investigation was to determine the EFA requirement for swine by the same method previously applied to rats, and to learn whether the relationship of tissue polyunsaturated acids to dietary linoleate is a general phenomenon or pertains only to rats.

EXPERIMENTAL

Forty-nine miniature swine were obtained by hysterectomy from the Hormel Foundation herd. This procedure provided experimental animals which had not received colostrum or sow's milk, thereby assuring that they had minimal reserves of EFA at the beginning of supplementation. These pigs were reared in isolation units, fed fortified cow's milk diets for several days, and then fed dry purified diets beginning at ages ranging from one to three weeks. Data from 17 of 22 animals treated similarly and reported earlier (Hill et al., '57) are also included here, the other 5 being omitted because of inadequate information about dietary linoleate intake or too short a time on experiment.

Composition of the purified diets used is shown in table 1.[2] Diets were mixed twice weekly, stored at $-20°C$, and fed ad libitum until each pig consumed 1 kg per day, the maximum allowed any animal. Basal diets were supplemented with ethyl linoleate, a urea complex of ethyl linoleate, or corn oil. Lipids in the diets were extracted in a Soxhlet apparatus and the lipid was analyzed by alkaline isomerization for polyunsaturated fatty acid (PUFA) content (Holman and Hayes, '58). The amount of dienoic fatty acid measured by isomerization and that of the supplement was used to calculate dietary linoleate as percentage of calories. The

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TABLE 1
Composition of purified diets

Ingredient, %	Diet number						
	5	7	16	17	18	19	20
Glucose[1]	56.2	56.4	66.1	71.0	71.6	65.0	34.4
Casein[2]	20.0	20.0	20.0	20.0	16.0	16.0	—
Gelatin	8.0	8.0	4.0	—	—	—	—
Skim milk, spray-dried	—	—	—	—	—	—	50.0
α-Cellulose	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Beef tallow	—	—	—	—	—	10.0	10.0
Lard, stripped	—	—	—	—	4.0	—	—
Hydrogenated coconut oil[3]	5.0	5.0	—	—	—	—	—
Methionine	0.3	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol	0.5	0.5	0.5	0.5	—	—	—
Urea[4]	—	—	0.4	0.4	—	—	—
Chlortetracycline	0.02	0.02	—	—	—	0.01	0.01
K_2HPO_4	0.6	0.6	—	—	—	—	—
$CaHPO_4$	3.5	3.5	2.0	2.0	2.0	2.0	—
KCl	—	—	0.5	0.5	0.5	0.5	—
NaCl	0.5	0.5	0.5	0.5	0.5	0.5	—
$MgSO_4 \cdot 7H_2O$	0.04	0.04	0.5	0.5	0.5	0.5	0.2
Trace minerals[5]	+	+	+	+	+	+	+
Vitamins[6]	+	+	+	+	+	+	+
Calories/kg	3605	3605	3410	3482	3709	4000	4000

[1] Cerelose, Corn Products Refining Company, New York.
[2] Vitamin-Test Casein, Nutritional Biochemicals Corporation, Cleveland.
[3] Durkee Company.
[4] EFA supplements to rations 16 and 17 were made as urea complexes.
[5] Trace mineral mix supplied the following per kg of ration: (in milligrams) $MnSO_4 \cdot 4H_2O$, 250; $ZnSO_4 \cdot H_2O$, 200; KI, 40; $CuSO_4 \cdot 5H_2O$, 40; $CoSO_4 \cdot 7H_2O$, 20; and (in grams) $FeSO_4 \cdot 7H_2O$, 1.
[6] Vitamin mix supplied the following per kg of ration: (in milligrams) biotin, 0.5; thiamine·HCl, 11; Ca pantothenate, 55; riboflavin, 22; pyridoxine·HCl, 22; niacin, 110; folic acid, 2; menadione sodium bisulfite, 2; *d*-α-tocopheryl acetate, 100; and also choline chloride, 2 gm; vitamin B_{12}, 110 μg; vitamin A (as palmitate), 9000 I.U.; and vitamin D_2, 900 I.U.

amounts of other polyunsaturated acids in the diets were negligible.

Animals were sacrificed by CO_2 anesthesia and exsanguination, tissues extracted with ethanol-ether (3:1), and lipids analyzed for polyunsaturated fatty acids by alkaline isomerization. Tissue samples were also stored in formalin for histological examination.

Seven of the first 17 animals fed diets 5 and 7 failed to live the length of the experiment (Hill et al., '57), causing a variation of supplementation of 31 to 98 days. Of the additional 49 young swine reported here, all lived to complete the experiments, which for 47 pigs varied from 56 to 77 days. Two pigs fed 0.87% of calories as linoleate were maintained with the diet for 701 and 799 days, and are included to show the results of feeding a purified diet for an extended time.

In table 2 are shown the diets fed, level of dietary linoleate, duration of the period of supplementation, number of pigs fed each level, weight gains, and the results of tissue lipid analyses. Seven basal diets were employed using lard, tallow or hydrogenated cottonseed oil as fat, and providing 13 different dietary levels of linoleate ranging from 0 to 12.9% of calories.

The experimental design revealed in this report is a compromise between opportunity and plan, and its deviations from perfection will be apparent to the purist. Prior to the studies in rats (Holman, '60) which reported the relationship between dietary linoleate and tissue triene:tetraene ratio, a number of analyses had been made on tissues from swine fed a few known levels of linoleate. When the relationship became known, it was thought advisable to interpret the data for the older swine in the new light, and additional swine were therefore fed other known levels of linoleate to fill in the gaps in the curve to

TABLE 2

Weight gain and polyunsaturated fatty acid content of heart and liver lipids

Dietary linoleate[1]	Days on exp.[2]	Av. weight gain/day	Polyunsaturated fatty acids as % of total lipid extracted				
			Dienoic	Trienoic	Tetraenoic	Pentaenoic	Hexaenoic
% of cal.		*gm*					
					Heart		
0.00–0.02[5,7,16,17]	31–98(25)	117 ± 13[3]	2.91 ± 0.35	8.75 ± 0.89	1.93 ± 0.15	0.33 ± 0.03	0.21 ± 0.02
0.23–0.27[5,19]	56–84 (9)	207 ± 19	7.42 ± 1.05	6.15 ± 0.53	3.51 ± 0.19	0.92 ± 0.21	0.22 ± 0.06
0.43[20]	56 (3)	203 ± 19	11.96 ± 0.81	4.57 ± 0.71	5.77 ± 0.95	1.59 ± 0.08	0.48 ± 0.05
0.63[20]	56 (3)	194 ± 12	15.15 ± 0.94	3.77 ± 0.36	6.98 ± 0.24	1.77 ± 0.06	0.35 ± 0.21
0.83[20]	56 (3)	186 ± 32	14.33 ± 0.07	2.83 ± 0.17	7.19 ± 0.88	1.46 ± 0.07	0.69 ± 0.06
0.87[18]	701, 799(2)	116 ± 7	6.85 ± 1.65	0.08 ± 0.08	14.73 ± 0.12	1.68 ± 0.01	0.31 ± 0.06
1.24[20]	56 (3)	185 ± 11	15.27 ± 0.87	2.87 ± 0.58	7.65 ± 0.46	1.62 ± 0.16	0.63 ± 0.14
1.48[20]	56 (3)	177 ± 17	14.51 ± 0.57	1.87 ± 0.07	7.54 ± 0.52	1.39 ± 0.12	0.56 ± 0.06
1.75[20]	56 (3)	192 ± 22	14.88 ± 0.41	1.58 ± 0.12	8.16 ± 0.53	1.39 ± 0.08	0.52 ± 0.04
2.43[19]	56 (3)	279 ± 7	12.33 ± 1.73	1.14 ± 0.23	11.09 ± 0.73	1.50 ± 0.08	0.28 ± 0.03
4.64[19]	56 (4)	283 ± 4	14.75 ± 1.76	0.86 ± 0.17	11.22 ± 1.85	1.26 ± 0.16	0.26 ± 0.06
6.84[19]	56 (3)	261 ± 45	17.44 ± 0.46	0.70 ± 0.11	10.51 ± 0.80	1.13 ± 0.11	0.21 ± 0.06
12.92[17]	69 (2)	184 ± 66	23.35 ± 1.45	0.82 ± 0.00	11.37 ± 0.43	1.11 ± 0.02	0.10 ± 0.03
					Liver		
0.00–0.02[5,7,16,17]	31–98(25)	117 ± 13[3]	1.65 ± 0.20	7.49 ± 0.54	2.03 ± 0.20	0.25 ± 0.02	0.24 ± 0.03
0.23–0.27[5,19]	56–84 (9)	207 ± 19	3.65 ± 0.37	5.81 ± 0.26	4.64 ± 0.46	1.06 ± 0.26	0.58 ± 0.21
0.43[20]	56 (3)	203 ± 19	5.76 ± 0.21	4.66 ± 0.41	6.52 ± 0.48	1.72 ± 0.14	1.07 ± 0.07
0.63[20]	56 (3)	194 ± 12	7.89 ± 0.18	4.02 ± 0.24	9.25 ± 0.31	2.07 ± 0.06	1.30 ± 0.32
0.83[20]	56 (3)	186 ± 32	7.37 ± 0.36	3.42 ± 0.33	8.94 ± 0.88	1.63 ± 0.08	1.03 ± 0.18
0.87[18]	701, 799(2)	116 ± 7	5.58 ± 0.03	0.86 ± 0.55	17.25 ± 0.55	2.16 ± 0.36	0.63 ± 0.44
1.24[20]	56 (3)	185 ± 11	8.06 ± 0.35	2.78 ± 0.20	10.85 ± 0.71	1.66 ± 0.16	1.13 ± 0.22
1.48[20]	56 (3)	177 ± 17	8.21 ± 0.48	2.83 ± 0.32	9.77 ± 0.57	1.44 ± 0.04	0.85 ± 0.13
1.75[20]	56 (3)	192 ± 22	9.73 ± 0.47	2.30 ± 0.10	9.50 ± 0.83	1.34 ± 0.12	0.73 ± 0.07
2.43[19]	56 (3)	279 ± 7	9.72 ± 0.68	1.55 ± 0.00	11.97 ± 0.47	1.58 ± 0.12	0.69 ± 0.08
4.64[19]	56 (4)	283 ± 4	14.42 ± 0.82	1.85 ± 0.20	12.12 ± 0.57	1.40 ± 0.07	0.58 ± 0.10
6.84[19]	56 (3)	261 ± 45	16.60 ± 1.57	1.31 ± 0.17	12.21 ± 1.49	1.46 ± 0.22	0.47 ± 0.10
12.92[17]	69 (2)	184 ± 66	20.15 ± 0.73	2.24 ± 0.04	13.85 ± 0.52	1.44 ± 0.14	0.32 ± 0.06

[1] Superscript numbers indicate the diets used at each level of linoleate.

[2] Number of pigs indicated in parentheses.

[3] Standard error of the mean.

be described. This resulted in a collection of data from several experiments, in which there were a number of minor variables, but in which the amount of dietary linoleate was known. Despite the heterogeneous nature of the experiments, however, the authors feel the results are so clear-cut that the data should be reported, and that the phenomena involved are adequately described to allow estimation of the EFA requirement of swine. In fact, the heterogeneous nature of the experiments and the uncontrolled variables are the strongest argument that the phenomenon to be described is controlled by dietary linoleate rather than by these many variables, and that it is valid for assessing EFA requirement and status.

RESULTS AND DISCUSSION

When average weight gains for the groups were plotted against linoleate intake, the curve appeared to be biphasic. The lowest linoleate supplement (0.23% of calories) produced a marked increase in rate of gain, but the next few increments, through 1.75% of calories, did not improve the rate of gain. A further increase in rate of gain was achieved by those groups which were given supplements of 2.43% of calories or more. Rate of gain of the unsupplemented group was significantly different from that of the group fed 0.23 to 1.75% of calories ($P < 0.001$). The latter was, in turn, significantly different from the rate of those fed 2.43% of calories or more ($P < 0.001$). The apparent biphasic nature of the curve relating rate of gain to linoleate intake is not understood, but it is clear that an amount of linoleate equal to 2.43% of calories meets the pigs' requirement for growth. The two pigs fed the 0.87% linoleate calories were 701 and 799 days old at termination of the experiment, and were far past the rapid growth stage of the other pigs. This accounts for the much lower growth rate of these older pigs compared with others fed levels of dietary linoleate under 2% of calories.

As shown in table 2, the pigs fed low dietary levels of linoleate had low levels of dienoic and tetraenoic fatty acids and high levels of trienoic fatty acids in heart and liver lipids. This relationship rapidly became reversed as the dietary level of linoleate was increased. This confirms our earlier results with swine tissue lipids, as well as results of several other investigators who used rats.

When the ratio of trienoic to tetraenoic fatty acids in lipids was plotted, as in the study with rats (Holman, '60), almost identical curves were obtained for liver and heart, with the ratios falling rapidly as dietary linoleate increased to a level of about 0.75% of calories (fig. 1). This was followed by rapid changes in the slopes of the curves which became essentially constant when the level of approximately 2% of calories was reached. These data and the growth data indicate that the minimum dietary linoleate requirement for swine is near 2% of the total calories.

One striking fact in this study is that the relationship between the triene/tetraene ratio of tissue lipids and dietary linoleate is consistent, despite variations in diet composition, kind and amount of dietary fat, duration of supplementation, and sex, age and genetic strain of animals. Some of these variables have a minimal effect upon the phenomenon, but do not obscure the relationship. For example, total saturated dietary fat content has been shown to have an influence upon EFA requirement and the triene/tetraene ratio (Peifer and Holman, '59). In the present study the deviation of the two swine fed 0.87% of calories as linoleate, from others fed similar levels of linoleate could be caused by the extended time of supplementation and their greater age.

Although Witz and Beeson ('51) reported dermatitis in 8 swine fed a fat-free purified diet, they made no specific attempt to study EFA involvement. Leat ('59) fed diets containing fish meal, dried skim milk, extracted palm kernel cake, brewers' yeast and cassava to weanling swine. He observed mild dermatitis in 4 animals which were unsupplemented but no dermatitis in the 4 which were given 2 ml of olive oil per 100 gm of diet. Leat concluded that, under his conditions, a dietary level of 0.03% of linoleic acid may meet the requirement for normal growth but is marginal for normal skin development. In our present experiments in which the piglets were taken by hysterec-

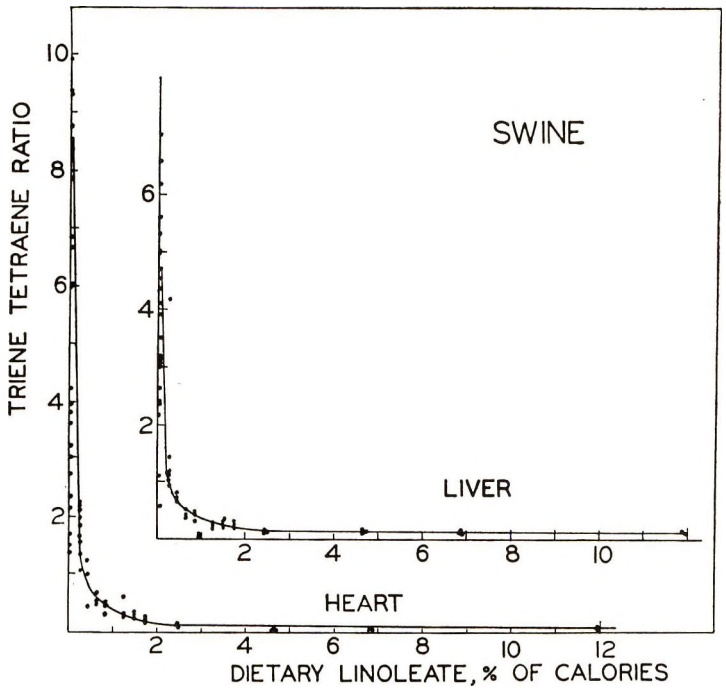

Fig. 1 Relationship of triene/tetraene ratio of liver and heart lipids to dietary linoleate in a population of 66 swine.

tomy and reared without access to colostrum or sow's milk, no dermatitis was observed in the unsupplemented group of 25 animals. We have no explanation for this difference in results between laboratories unless it could be traceable to relative humidity, which is known to affect the degree of dermatitis in EFA-deficient rats. The biochemical lesion (abnormal PUFA content of tissue lipids) has been shown to accompany classical EFA deficiency in rats and it has been found to be more regular and less influenced by external conditions than is the dermatitis. Therefore, we believe that the biochemical lesion should take precedence over dermatitis in judging EFA deficiency. On this basis, and on the basis of weight gains, the unsupplemented swine in this study are judged to be deficient in EFA.

Hill et al. ('57) reported calcification of the media of aortas of EFA-deficient pigs fed diets 5 and 7. None of the 49 pigs examined in the present studies had aortic lesions. The only major difference in the diets of these groups was the amount of magnesium added, which was increased from 40 to 495 mg per kg. Mayo et al. ('57) reported the magnesium requirement of young swine to be from 270 to 400 mg per kg. Our earlier diets 5 and 7, therefore, were borderline, if not actually deficient, in magnesium. Since Moore et al. ('38) reported calcification of the media of the aorta of dairy calves deficient in magnesium, and more recently the work of Vitale et al.[3,4] showed the same symptom in rats and in dogs, it was likely that the earlier aortic lesions were induced by a magnesium deficiency. The pigs showing aortic lesions, however, were among those fed EFA-deficient diets. None of those supplemented even at the low level of 0.23 to 0.27% of calories showed aortic lesions, indicating that in EFA deficiency the additional relative magnesium

[3] Vitale, J. J., E. E. Hellerstein, M. Nakamura, B. Lown, D. M. Hegsted and J. Caner 1959 Arteriosclerosis in puppies fed a magnesium deficient diet. Federation Proc., *18:* 550 (abstract).

[4] Vitale, J. J., P. L. White, M. Nakamura and D. M. Hegsted 1957 Effect of feeding an atherogenic diet on magnesium requirement. Federation Proc., *16:* 400 (abstract).

deficiency may have more serious consequences.

When linear regression equations were calculated for the two legs of the curves pictured in figure 1, the following equations were obtained:

Swine heart

Horizontal leg : $X = 5.2650 - 10.4130Y$
Vertical leg : $X = 0.3050 - 0.0445Y$

Swine liver

Horizontal leg : $X = 5.9250 - 12.4072Y$
Vertical leg : $X = 0.3557 - 0.07257Y$

where X = dietary linoleate as percentage of calories and Y = triene/tetraene ratio. For each tissue the point of intersection of the two lines was calculated. The dietary linoleate value at this intersection was found to be 0.28 and 0.32% of calories for heart and liver, respectively. Similar calculations for curves described previously (Holman, '60) for rats yielded points of intersection at 0.40, 0.37 and 0.27% of calories for heart, plasma and erythrocytes, respectively. Within the limits of experimental uncertainty ($\sigma = 16\%$ relative error) the requirements of the two species are identical when expressed by this means. Although these values are not offered as minimum linoleate requirements, this mode of expression is the most precise method available to us for the comparison of the relationships existing in different tissues and species. One might predict that the lipids from other tissues and other animals will show similar values. This means of comparison is more precise than many means of nutritional evaluation, and might find application in studies of other nature.

The minimum requirement can be deduced from the analytical data in several arbitrary ways. In the preceding paragraph a point of intersection of the linear portions of the two legs of the curves occurred at about 0.30% of calories. The point at which the slope of the curve changes most rapidly is at about 1% of calories, and this has been used previously to express the requirement of rats and human infants (Holman, '60a). If, however, a level of dietary linoleate is sought at which the slope of the curve becomes essentially constant, the requirement must be placed near 2% of calories. This value is favored because it is also indicated from growth data. The multifunctional nature of EFA requirement is also indicated from other points of view in studies which will be reported in a following paper.

It should be emphasized in conclusion that the dependence of tissue PUFA upon dietary linoleate is a phenomenon which is little affected by many variables. Despite three levels of total fat in the diet, three kinds of dietary fat, some variaton in duration of supplementation, and differences in age, strain and sex of the animals, the relationship of triene/tetraene ratio to dietary linoleate is unmistakable and the variability remarkably low. Undoubtedly, if these several variables could be controlled, the scatter of points would be less, but even so, the data suffice for stating the EFA requirement on the basis of a definite biochemical lesion.

SUMMARY

Sixty-six swine were fed purified diets varying in linoleate content from zero to 12.9% of calories. Polyunsaturated fatty acids were determined in the lipids of the hearts and livers. Characteristic high levels of tissue trienoic acids and low levels of tetraenoic acids were observed in the unsupplemented swine. As dietary linoleate was increased, this relationship was rapidly reversed. The linoleate requirement was deduced from the plot of triene/tetraene ratio versus dietary linoleate. The curves for heart and liver were remarkably similar. From these curves and the weight gains, the dietary linoleate requirement is stated to be near 2% of calories.

ACKNOWLEDGMENTS

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Invitation for Nominations for 1962 American Institute of Nutrition Awards

Nominations are requested for the 1962 annual awards administered by the American Institute of Nutrition to be presented at the next annual meeting. Nominations may be made by anyone, including members of the Nominating Committees and non-members of the Institute.

The following information must be submitted: (1) Name of the award for which the candidate is proposed. (2) A brief convincing statement setting forth the basis for the nomination. A bibliography and supporting letters are not to be submitted. (3) Five copies of the nominating letter must be sent to the chairman of the appropriate nominating committee *before October 1, 1961*, to be considered for the 1962 awards.

General regulations for A.I.N. awards. Membership in the American Institute of Nutrition is not a requirement for eligibility for an award and there is no limitation as to age. An individual who has received one Institute award is ineligible to receive another Institute award unless it is for outstanding research subsequent to or not covered by the first award.[1] A Jury of Award composed of A.I.N. members, which makes final selection and remains anonymous, may recommend that an award be omitted in any given year if in its opinion the work of the candidates nominated does not warrant the award. An award is usually given to one person but, if circumstances and justice so dictate, a Jury of Award may recommend that any particular award be divided between two or more collaborators in a given research.

Presentation of awards will be made at the annual dinner at the annual meeting.

1962 Borden Award in Nutrition

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

Former recipients of this award are:

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

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1962 Osborne and Mendel Award

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

[1] Including recipients of the former Mead-Johnson award. These are listed at the end of this notice.

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

Former recipients of this award are:

1949 – W. C. Rose
1950 – C. A. Elvehjem
1951 – E. E. Snell
1952 – Icie Macy Hoobler
1953 – V. du Vigneaud
1954 – L. A. Maynard
1955 – E. V. McCollum
1956 – A. G. Hogan
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Former recipients of the Mead-Johnson Award presented by A.I.N. are:

1939, C. A. Elvehjem; 1940, W. H. Sebrell, Jr., J. C. Keresztesy, J. R. Stevens, S. A. Harris, E. T. Stiller, and K. Folkers; 1941, R. J. Williams; 1942, G. R. Cowgill; 1943, V. du Vigneaud; 1944, A. G. Hogan; 1945, D. W. Woolley; 1946, E. E. Snell; 1947, W. J. Darby, P. L. Day and E. L. R. Stokstad; 1948, F. Lipmann; 1949, Mary S. Shorb and K. A. Folkers; 1950, W. B. Castle; 1951, no award; 1952, H. E. Sauberlich.

Invitation for Nominations for 1962 American Institute of Nutrition Fellows

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

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

Nominations (in 5 copies) are due by October 1. A supporting statement giving the reason for the nomination is desirable but not necessary.

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

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David B. Hand
Icie Macy Hoobler
H. E. Robinson

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

Thorne M. Carpenter (1958)
George R. Cowgill (1958)
Eugene F. DuBois (1958)
R. Adams Dutcher (1961)
Ernest B. Forbes (1958)
Casimir Funk (1958)
Albert G. Hogan (1959)
Icie Macy Hoobler (1960)
Paul E. Howe (1960)
Leonard A. Maynard (1960)
Elmer V. McCollum (1958)
Harold H. Mitchell (1958)
Agnes Fay Morgan (1959)
John R. Murlin (1958)
Helen T. Parsons (1961)
William C. Rose (1959)
Arthur H. Smith (1961)
Harry Steenbock (1958)
Robert R. Williams (1958)