ERRATUM

Bressani, R. 1962 Effect of amino acid imbalance on nitrogen retention. I. Effect of a relative deficiency of tryptophan in dogs. J. Nutrition, *78:* 365.

This notice refers to errors in the ratios given in the last paragraph of the Discussion and in the Summary.

The methionine-to-tryptophan ratio when methionine was omitted from the basal diet is 1.33/1, while the M/T ratio in the basal diet is 2.30/1.

Protein, Carbohydrate and Fat Content of the Diet of the Rat as Related to Growth

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In the course of earlier experiments concerned with the amino acid supplementation of corn, rice and wheat-base diets, it was observed in this laboratory that the inclusion of dietary fat invariably decreased the rate of growth that these diets would support in the laboratory rat. Since Mendel ('23) had reported an animal ate first to satisfy his energy needs, it was initially assumed that the higher caloric density of the fat-containing diet had reduced the food intake causing a decreased protein consumption by animals already receiving a low protein diet with a resultant decrease in growth rate.

Later, however, while determining the protein efficiency ratio of certain foodstuffs, it was found that no growth retardation occurred with a low protein diet upon the addition of fat if glucose rather than corn starch was the carbohydrate source. Reported herein are some of the results obtained in exploring this interesting phenomenon.

METHODS AND RESULTS

Male weanling rats of the Holtzman strain were randomly divided into groups of 10, individually housed on screen and allowed to feed ad libitum. All diets, in addition to varying amounts of protein, carbohydrates and fat, contained 5% cellulose, 4% salt mixture (Wisconsin no. 4), 1% corn oil as a source of essential fatty acids, and a vitamin addendum supplying the following micronutrients per 100 gm of food: In milligrams: thiamine·HCl, 1.0; riboflavin, 2.0; pyridoxine·HCl, 1.0; calcium D-pantothenate, 10.0; niacinamide, 10.0; inositol, 5.0; choline as bitartrate, 100.0; *p*-aminobenzoic acid, 30.0; biotin, 0.05; folic acid, 0.2; α-tocopherol, 14.2; menadione, 14.2; vitamin B_{12} triturate (0.1% trituration with mannitol), 10.0. In units: calciferol, 300; vitamin A palmitate, 1600.

The animals were weighed at 5-day intervals and weekly food consumptions were determined.

In table 1 are shown the weight gains in 8 separate experiments in which a comparison was made of the effect of glucose and starch when used with diets containing 10% casein and either 1 or 10% corn oil. In 7 of the 8 experiments in the presence of starch, increasing the corn oil content of the diet depressed the growth rate. In the presence of 1% corn oil the average growth rate of the starch-fed animals was 98.3% of that of those receiving glucose, whereas in the presence of 10% corn oil this percentage dropped to 75.5%. ($P = 0.01$ by analysis of variance; Snedecor, '56.) In a more limited investigation sub-

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TABLE 1

Rate of growth of weanling rats on a 10% casein diet as influenced by corn oil and different carbohydrate sources

Dietary carbohydrate	Dietary fat	28-Day weight gain in grams											
		Exp. 1	2	3	4	5	6	7	8	9	10	11	Avg
Starch	1% corn oil	105	99	106	101	108	116		96	125			107
Glucose	1% corn oil		105	111	109	114	105		123	95			109
Starch	10% corn oil	79	92	82	79	72	116	71	86	70	73	61	80[1]
Glucose	10% corn oil	113	105	123	94	94	104	106	117	104	104	105	106

[1] $P = 0.01$ by analysis of variance, (Snedecor, '56).

TABLE 2

Effect of protein on rate of growth of weanling rats receiving corn oil and starch or glucose

Diet	28-Day average weight gain	
	Glucose	Starch
	gm	*gm*
10% Casein, 1% corn oil	109	111
10% Casein, 10% corn oil	110	78
15% Casein, 1% corn oil	142	145
15% Casein, 10% corn oil	154	152
20% Casein, 1% corn oil	160	158
20% Casein, 10% corn oil	167	176
10% Casein, 0.2% DL-methionine, 10% corn oil	132	142
10% Casein, 0.2% L-cystine, 10% corn oil	140	142
10% Casein, 10% glutamic acid, 10% corn oil	84	57
10% Wheat gluten, 0.2% L-lysine·HCl, 10% corn oil	50	27
10% Wheat gluten, 0.4% L-lysine·HCl, 0.3% DL-threonine, 10% corn oil	106	88
10% Whole egg protein (supplies 9% fat)	129	135

TABLE 3

Effect of various fats on growth of weanling rats when fed glucose or starch with a 10% casein diet[1]

Dietary fat	Weight gain 28 days			
	Experiment 1		Experiment 2	
	Glucose	Starch	Glucose	Starch
None	109	101	105	116
9% Hydrogenated coconut oil[2]	95	86	100	94
9% Olive oil	99	81	105	100
9% Safflower oil	101	97	98	89
9% Linseed oil	80	82	87	80
9% Cod liver oil	79	63	85	72

[1] Basal diet contained 1% corn oil as a source of essential fatty acids.
[2] Hydrol, Durkee's Famous Foods, Chicago, Illinois.

stitution of sucrose for starch caused an even poorer performance.

Shown in table 2 are the effects of variations in protein intake. Increasing the casein content of the diet, supplementing with DL-methionine or L-cystine, or replacing casein with an isonitrogenous quantity of whole egg protein appears to eliminate the differences observed with glucose and starch. On the other hand, addition of glutamic acid as a nonspecific source of nitrogen or substitution of wheat gluten whether or not fortified with the first or both of its two most limiting amino acids, lysine and threonine did not eliminate the differences between the two sources of carbohydrate. It appears that any change in protein which caused the growth rate to approach a maximum tends to cancel out the differences observed with glucose and starch.

Recorded in table 3 are the data obtained by substituting a variety of fats for the corn oil in the 10% casein diet. While the differences are small, in almost all cases the starch supported a less rapid growth rate than did glucose. It is of some interest that the more highly unsaturated fats depressed growth of the animals receiving glucose, an effect not observed when using diets with more adequate protein.

The food consumption data collected during these experiments show that when growth inhibitions occurred there was also a decrease in food intake. With the starch-containing diets the feed efficiency was calculated to be about 85% as great as with those containing dextrose. While this is a significant difference it is probably no greater than might be expected under

TABLE 4

Effect of antibiotics on growth rate of rats receiving diets containing 10% casein, 10% corn oil and starch or glucose

Dietary antibiotic	28 Day average weight gain	
	Glucose	Starch
	gm	*gm*
None	100	76
0.1% Neomycin	103	58
0.2% Neomycin	106	79
0.4% Neomycin	110	79
0.2% Oxytetracycline	109	90
0.4% Oxytetracycline	114	85
0.2% Chlortetracycline	115	104
0.4% Chlortetracycline	113	107
2.0% Succinylsulfathiazole	107	88
0.2% Penicillin	101	90
0.2% Streptomycin	102	78

conditions in which a higher percentage of the intake must be used for maintenance.

One of the more obvious means by which starch and fat might effect a decreased food consumption and a concomitant decreased growth rate is through an effect on the bacterial flora of the gut. To test this hypothesis a number of antibiotics were fed with the diets containing 10% casein, 10% corn oil and glucose or starch. The results are shown in table 4. Chlortetracycline almost completely eliminated the difference in growth rate between starch- and glucose-fed rats; oxytetracycline, penicillin and succinylsulfathiazole were much less effective in this respect; whereas neomycin and streptomycin had no effect. Of the antibiotics used only the tetracyclines caused an increase in growth rate of both starch- and glucose-fed rats ($P = 0.05$).

DISCUSSION

A profound effect on food consumption and consequently on growth rate has been shown to involve all three of the major foodstuffs. This is another demonstration of the close interrelationship of all nutritional factors. The mechanism of the effect observed is not known but it may be mediated through the bacterial flora of the gastrointestinal tract. If so, the effect appears to be due to production of an anorexic agent rather than to competition for nutrients since it is accompanied by a decrease in food consumption and since neomycin which is widely used for essentially complete elimination of bacteria from the gut did not eliminate the difference in performance of animals on the starch and glucose containing diets. The reason for the relative ineffectiveness of oxytetracycline is not known. However, a difference in effect of the two tetracycline derivatives on plasma cholesterol in another species was observed in these laboratories (Howe and Bosshardt,'60).

If growth inhibition is due to an anorexic agent, how then does increasing the quantity or quality of the protein overcome its effect? Presumedly, either by a direct effect by allowing overgrowth of the elaborating bacteria or by increasing the detoxifying capacity of the host. Pecora ('53) has reported that both penicillin and chlortetracycline accelerated the growth rate of rats fed a rice diet supplemented with lysine and threonine. Penicillin, however, was more effective which is the reverse of what was observed in these experiments.

The lower food consumption observed with the starch diet could be due entirely to palatability. If so, it remains unexplained why chlortetracycline or an increase in protein quality should improve the diet in this respect.

Two similar methods for the determination of protein value of foodstuffs have recently been reported (Chapman et al., '59; Derse, '60). Since these procedures are both based on rat growth experiments using fat-containing diets high in starch or sucrose and low in protein, it appears likely that inclusion of chlortetracycline in these diets might yield more consistent results.

Whether the interrelationships described herein exist in man is unknown, but the experiments of Scrimshaw and Guzman ('53) in which chlortetracycline was found to cause an increase in both height and weight of Guatemalan children receiving a low protein diet, suggest that they may.

SUMMARY

1. Addition of 9% corn oil to the diet of rats receiving suboptimal protein and corn

starch as a source of carbohydrate decreased food consumption and depressed growth. These effects were not observed if glucose replaced starch in the diet.

2. Improvement of the protein of the diet quantitatively or qualitatively eliminated the difference in performance of animals receiving starch and glucose.

3. Replacement of corn oil by other fats caused growth depression of rats receiving a starch diet. Linseed oil and cod liver oil also caused a growth depression in glucose low-protein diets.

4. Addition of chlortetracycline eliminated the growth-depressing effect of adding corn oil to the diets of low-protein starch fed rats. Oxytetracycline, penicillin, neomycin, streptomycin and succinylsulfathiazole were less effective or without effect.

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Nitrogen Excretion in Sweat and Its Relation to Nitrogen Balance Requirements

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It has been generally observed that the rate of sweating is dependent on exposure to heat, physical exercise and also on the emotional state, which will stimulate the sweat excretion by the apocrine glands (Cuthbertson and Guthrie, '34). The chemical properties of sweat depend primarily (a) on acclimatization to heat; (b) on the rate of sweating; (c) on inherent characteristics of the individual; and (d) on many other factors (Dill et al., '38).

The minimal protein requirements are based on reports in the literature of investigations on nitrogen balance or equilibrium studies. In general, nitrogen balance losses have included only the urine and fecal excretions, although nitrogen losses are also observed in sweat and can possibly be found in expired air. Even though information on the dermal nitrogen losses has been published prior to 1950 (Mitchell and Hamilton, '49) neither the National Research Council's Food and Nutrition Board publication ('58) or the Food and Agriculture Organization of the United Nations publication ('57), mention the possibility of increased protein allowances under conditions that produce profuse sweating. These nitrogen losses in sweat might be of consequence in evaluating protein requirements, especially in hot humid, or hot dry areas, of individuals consuming low protein intakes.

In comprehensive reviews of the literaature, it has been reported by Cuthbertson and Guthrie ('34), Mitchell and Hamilton ('49) and more recently by Mitchell and Edman ('62), that dermal nitrogen losses are quite appreciable, ranging from 21 to 130 mg/100 ml of sweat. Mitchell and Hamilton ('49) observed that with a daily intake of 98 gm of protein, the nitrogen excretion in sweat, of men sweating profusely, averaged 152 mg/hour, which was approximately 22.5% of the total daily output. These authors also observed that during normal or minimal sweating conditions, the nitrogen loss in sweat averaged 15 mg/hour, equivalent to 2.7% of the total daily output. In general these dermal losses have been ignored in most of the nitrogen equilibrium studies reported in the literature.

These fairly high nitrogen excretions in sweat, especially during profuse sweating conditions, indicate that such losses are sufficiently important to warrant further investigation. It is the opinion of investigators (Mitchell and Hamilton, '49; Mitchell and Edman, '62) that these losses should be included as part of the total daily output in balance studies, otherwise erroneous conclusions can be drawn.

Two studies were designed in an attempt to answer some of the following questions: (a) what are the nitrogen losses in sweat of men performing fairly light and moderate, controlled physical activities at high environmental temperatures; (b) what are these losses in relation to the nitrogen intake and nitrogen balance; (c) what are these losses in relation to protein requirements; and (d) do these nitrogen losses in sweat decrease appreciably during acclimatization?

EXPERIMENTAL DESIGN

Two studies were conducted during the months of July and August in 1960 and 1961 in Denver, Colorado. In the 1960 study the test periods were divided into three separate temperature phases of 21.1,

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29.4 and 37.8°C (70, 85 and 100°F), each of 4 days' duration. Each phase was repeated randomly 4 times for a total of 48 days. The test group consisted of 8 healthy men between the ages of 19 and 25. The activity level of the men during all the test periods was controlled at a constant daily rate. The physical activity included riding a bicycle ergometer for 50 minutes a day at a moderately high activity level (1.2 to 1.6 liters of oxygen/minute) that produced 120 watts of work, and riding an ergometer for 50 minutes a day at a moderate rate (0.6 to 0.9 liters of oxygen/minute).

In 1961 the study was conducted for a total of 32 days on three healthy young men. The study consisted of an 8 day preliminary period (1) at 24°C (75°F) and 50% relative humidity (RH), four 4-day periods at 37.8°C (100°F) and 70% RH (2, 3, 4 and 5), and an 8-day recovery or adjustment period at 24°C (75°F) and 70% RH (6). The daily physical activity consisted of only 30 minutes of moderate activity on the bicycle ergometer, the remainder of the day being spent in sedentary-type activities. The subjects exercised in a random order each day and the exercise periods were alternated between mornings and afternoons. When the men left the testing area (evenings) no moderate-to-heavy activities were permitted. During the evening hours, the subjects were closely supervised by the Metabolic Ward personnel.

All of the test phases were performed in the environmental chamber from 8 AM to 4:30 PM daily with the exception of each third evening during periods 2, 3, 4 and 5 (1961 study) when the men slept overnight in the hot room. Nocturnal sweat samples, using the arm bag, were collected continuously, for 2- to 3-hour intervals to determine whether the sweat concentration of nitrogen was decreased with longer exposure to the heat.

The food was prepared by the dietitians and consisted of 4 diets that were rotated during each study. All food was offered and consumed at regular mealtimes and no other food was permitted during the day. Cool tap water (in individual weighed polyethylene bottles) was supplied ad libitum for drinking purposes and the fluid intake of each man was recorded daily. When the men left the immediate vicinity of the ward (movies, church) they carried their water bottles with them.

The sweat collection periods were performed for a total of 7.5 hours daily throughout the experiment, 3.5 hours in the morning and 4 hours during the afternoon periods. Sweat rates were measured for each period using the method of Adolph et al. ('47). These sweat rates were determined by measuring weight changes during the morning and afternoon periods and then adjusting for water intake, and for water loss in the urine and feces. (Gaseous exchange of respiration contributes a small quantity, which usually constitutes less than 1% of the observed decrease in body weight.) Sweat samples were collected daily during both the morning and afternoon periods. These samples were collected in polyethylene bags which covered one whole arm, and were representative for the entire period each subject was in the chamber. The arm and the bag were rinsed with distilled water and dried before each collection period began. But a question arises whether the comparison of arm sweat with total body sweat is valid. It has been shown by some investigators (Dill et al., '38; Johnston et al., '50; Ladell, '48) that the constitutents of arm sweat are reasonably representative of the total body sweat.

Complete urine and fecal collections were made for 4-day periods, coinciding with the repetition of the 4 menus used. Nitrogen balance was computed on each man for each period, based on the inclusion and exclusion of nitrogen in sweat as a source of nitrogen loss.

Chemical analyses of the food composites, urine, feces and sweat were performed for total nitrogen, urea nitrogen, ammonia, creatinine, and uric acid (Consolazio and Johnson, '60). The amino acid content of sweat was analyzed using the automatic amino acid analyzer (Spackman et al., '58).

RESULTS

In the 1960 study the nitrogen in sweat, during the daily 7.5-hour exposure in the environmental chamber, averaged 149, 189 and 241 mg/hour during the 70, 85 and

TABLE 1

Sweat rates and sweat nitrogen concentrations at various temperatures[1] (1960 and 1961 studies)

Environmental temperature	1960 Study			Days at 100°F	1961 Study		
	Sweat rate	Nitrogen in sweat			Sweat rate	Nitrogen in sweat	
°F	*gm/hr*	*mg/hr*	*mg/gm sweat*		*gm/hr*	*mg/hr*	*mg/gm sweat*
70	143	149	1.04[2]	1–4	310	310	1.00
85	242	189	0.78	5–8	350	219	0.63
100	312	241	0.77	9–12	382	207	0.54
				13–16	406	217	0.53

[1] Mean excretion of 8 men for 16 days in the 1960 study and mean excretion of three men for 4-day periods during the 1961 study.
[2] Only 10 samples analyzed at the 70°F temperature, but 52 samples at 85°F and 66 values at 100°F.

TABLE 2

Excretion of nitrogen and nitrogen compounds in sweat[1] per 7.5-hour exposure period (1960 and 1961 studies)

Environmental temperature	Total nitrogen	Ammonia	Urea nitrogen	Creatinine	Uric acid
°F	*mg*	*mg*	*mg*	*mg*	*mg*
Study 1, 1960					
70°	1118	34	526	11.6	—[2]
85°	1418	106	832	17.7	—[2]
100°	1808	238	1032	23.2	—[2]
Study 2, 1961					
100°, days 1–4	2379	209	—[2]	28.9	—
100°, days 5–8	1640	152	—[2]	25.2	3.1
100°, days 9–12	1551	145	—[2]	26.1	3.3
100°, days 13–16	1629	152	—[2]	24.3	2.9

[1] Mean excretion of 8 men for 16 days in the 1960 study and mean excretion of three men for 4-day periods during the 1961 study.
[2] No analysis.

100°F temperature phases, respectively (table 1). The sweat rates for the same periods averaged 143, 242 and 312 gm/hour during the daily chamber exposure. In this study the nitrogen excretion in sweat accounted for 9.2, 12.1 and 14.2% of the total nitrogen output for the same temperature phases.

The daily nitrogen intake for the 4 rotating menus averaged 14.90 gm (93.1 gm of protein) for study 1 and 13.63 gm (84.4 gm of protein) for the 1961 study.

The total nitrogen excreted in sweat by the three men during the 7.5 hours of exposure in the environmental chamber at 100°F was fairly constant after the first acclimatization period. During the last three periods the values ranged from 1.55 to 1.64 gm/man/period (table 2), equivalent to 219, 207 and 217 mg/hour.

The daily urinary excretion of nitrogen averaged 10.67 gm/day for the preliminary control period, 11.23, 11.09, 11.16 and 11.33 gm/day for the 4 consecutive periods at 100°F and 11.40 gm/day for the recovery or adjustment periods (table 3). The fecal nitrogen excretion rate was also constant during the 4 extremely hot periods. Nitrogen balance was computed during the study in two ways, exclusive of nitrogen losses in the sweat and with the inclusion of sweat nitrogen losses (table 3). The nitrogen balance of the periods at 100°F in the 1961 study, exclusive of the sweat nitrogen losses, were all positive, but when corrections were made for the nitrogen losses in sweat, all the balances at 100°F were on the negative side. In the 1960 study, the nitrogen balances were also positive, showing a

TABLE 3

Nitrogen balance in two studies, with and without the inclusion of sweat[1] *(1960 and 1961 studies)*

Environmental temperature	Intake	Output			Balance	
		Urine	Feces	Sweat[2]	Sweat excluded	Sweat included
°F	gm/man/day	gm/man/day			gm/man/day	
1960 Study						
85°	14.90	10.98	1.18	1.67	+2.74	+1.07
100°	14.90	10.95	1.37	2.06	+2.58	+0.52
1961 Study						
75°, control period	13.63	10.67	1.75	0.36	+1.21	+0.85
100°, days 1–4	13.63	11.23	1.78	2.63	+0.62	−2.01
100°, days 5–8	13.63	11.09	1.91	1.89	+0.63	−1.26
100°, days 9–12	13.63	11.16	1.95	1.80	+0.52	−1.28
100°, days 13–16	13.63	11.33	1.89	1.88	+0.41	−1.47
75°, recovery period	13.63	11.40	1.89	0.36	+0.34	−0.02

[1] Mean excretion of 8 men for 16 days in the 1960 study and mean excretion of three men for 4-day periods during the 1961 study. Periods at 75° were for 8-day periods.
[2] An assumed value of 15 mg/hour was included during the comfortable hours (3), based on local and prevailing conditions.

retention of + 2.74 and + 2.58 gm/man/day for exposures of temperatures at 85 and 100°F. Even after correction for the 7.5-hour sweat losses the nitrogen balances were still positive.

The data in table 4 represent the average diurnal variations in sweat nitrogen in milligrams per hour. The hourly variation is presented for days 7, 11 and 15 of exposure to 100°F. These values did not decrease appreciably after the first 4 days of acclimatization, which is the opposite that is found with calcium and other minerals excreted in sweat. Creatinine excretion in sweat ranged from 24.3 to 28.9 mg/period for the men living at 100°F, and showed no appreciable decrease in concentration from day to day or hour to hour (table 2). Creatinine and uric acid each accounted for approximately 1% of the total nitrogen.

As one would expect, the urea nitrogen concentration also increased with an increase in environmental temperature averaging 526, 832 and 1032 mg for the 7.5-hour exposure periods at 70, 85 and 100°F temperatures (table 2). Urea nitrogen excretions in sweat accounted for more than 50% of the total nitrogen excretion in sweat.

Values for the free essential and nonessential amino acids in sweat are presented in tables 5 and 6, respectively, for both studies. Of all the essential amino acids, lysine showed the greatest excretion, averaging between 147 to 195 mg,

TABLE 4

Diurnal and hourly variation of nitrogen in sweat; mean excretion of three men per interval (1961 study)

Time	Days at 100°F															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
hours[1]	mg/hour		mg/hour		mg/hour		mg/hour		mg/hour		mg/hour		mg/hour		mg/hour	
0–1.5							155				196					
1.5–3.5	361	381	335	299	201	153	160	255	158	209	200	224	127	254	—	261
4.5–6.5							234				208				230	
6.5–8.5	210	293	232	239	243	231	255	250	251	210	225	182	253	214	245	272
9.5–11							202				207				178	
11–13							203				194				185	
13–15							276				214				202	
15–23 (sleeping)							88				99				136	

[1] Zero time was 8 AM daily.

TABLE 5

Free essential amino acids composition of sweat;[1] mean excretion for three men during 7.5 hours' exposure to various temperatures (1960 and 1961 studies)

	1960 Study			1961 Study			
	Mean for 16 days			Days at 100°F			
	70°F	85°F	100°F	1–4	5–8	9–12	13–16
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Histidine	38	98	102	106	102	65	68
Isoleucine	5	16	20	36	22	16	16
Leucine	8	26	34	36	28	21	22
Lysine	54	143	178	218	195	147	165
Methionine	1	2	3	7	7	4	4
Phenylalanine	5	24	24	25	18	15	17
Threonine	15	10	10	86	66	50	50
Valine	9	30	40	43	36	27	26
Totals	135	349	411	557	474	345	368

[1] Sweat analyzed after centrifuging and discarding debris.

TABLE 6

Free nonessential amino acids composition of sweat;[1] mean excretion for three men during 7.5 hours' exposure to various temperatures (1960 and 1961 studies)

	1960 Study			1961 Study			
	Mean for 16 days			Days at 100°F			
	70°F	85°F	100°F	1–4	5–8	9–12	13–16
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Alanine	20	100	120	106	85	58	61
Arginine	8	19	28	23	33	Tr	Tr
Asparagine	103	357	443	571	448	318	417
Citrulline	12	42	56	61	47	34	32
Glycine	36	130	156	182	147	113	120
Glutamic acid	12	29	28	37	29	21	19
Proline	5	—	12	28	22	33	29
Serine	13	46	59	67	53	40	33
Tyrosine	12	40	46	52	43	29	32
Totals	221	763	948	1127	907	646	743

[1] Sweat analyzed after centrifuging and discarding debris.

after acclimatization to 100°F temperatures. In the study at three environmental temperatures, the lysine excretion averaged 54 mg at 70, 143 mg at 85, and 178 mg at 100°F for the 7.5-hour exposure periods. During a 7.5-hour collection period, lysine and threonine accounted for 27 and 11% of the total daily minimal requirement of essential amino acids. Asparagine accounted for approximately one-half of the total nonessential free amino acids in sweat. Fairly large quantities of alanine and glycine were also found in sweat (table 6). The total free amino acids excreted in sweat during the 7.5-hour exposure to 100°F temperatures, averaged more than 1 gm/period after acclimatization to heat. This was approximately 13% of the total nitrogen excreted in sweat.

In the 1960 study the ammonia excretion averaged 34, 106 and 238 mg for the 7.5 hour exposure periods at 70, 85 and 100°F, respectively (table 2). During the 16 days exposure to 100°F temperatures in the 1961 study, the excretion rates of ammonia averaged 209, 152, 145 and 152 mg for the 4 consecutive periods, showing a decrease in excretion with acclimatization to heat. These values, which averaged approximately 8.3% of the total nitrogen excreted, are considerably higher than the

blood levels of ammonia (Schwartz, '60). As expected, the ammonia excretion in sweat increased with an increase in sweat rate and environmental temperature.

DISCUSSION

It has again been observed in the present studies that there is an increase in sweat nitrogen excretion with an increase in environmental temperature, an increase of physical activity and an increase in sweat rate. Even though the sweat rates were increased during the warmer periods, the nitrogen concentration per gram of sweat was approximately the same at the 85 and 100°F temperatures.

In the 1961 study the nitrogen excretion in sweat decreased after the first 4 days of acclimatization to extreme heat. These values decreased to approximately 214 mg/hour, which were considerably higher than mean values of 152 mg/hour reported by Mitchell and Hamilton ('49). Decreased nitrogen excretion in sweat after acclimatization has also been observed by van Heyninger and Weiner ('52), Bass et al. ('55), Dill et al. ('38) and Mezinesco ('37). Sweat samples were collected continuously in the 1961 study for 24 hours during days 7, 11 and 15, when the men spent the day at 100°F. Sweat nitrogen output per hour did not change appreciably until after the men had retired and gone to sleep for the night. During the sleeping hours at 100°F (11 PM to 7 AM) the sweat rate (table 7) and the sweat nitrogen concentrations were decreased to approximately one-half of the day time rate, when the men were active and moving about. On arising the next morning the sweat rate and nitrogen concentration per hour had returned to the previous excretion level of 200 mg/hour.

Even under conditions of fairly high protein intakes, as in the 1961 study, the nitrogen balances were quite negative when the sweat losses were included. There seems to be no question that the nitrogen losses in sweat, under conditions that produce profuse sweating, will greatly affect the interpretation of nitrogen balance.

In the 1961 study the nitrogen in sweat losses based on a 7.5-hour collection period averaged 2.63, 1.89, 1.80 and 1.88 gm/day for the respective periods. In table 4 (considering days 7 and 11 only) when one computes the daily nitrogen loss for the time periods given (covering 21 hours) the nitrogen loss could be 3.50 and 3.48 gm/day for days 7 and 11. If a correction is also made for the other three hours, then a total 24-hour sweat nitrogen loss of 4.07 and 4.10 gm/days 7 and 11 is deduced. This appears extremely excessive until one recalls that Guthrie and Cuthbertson ('34) reported up to 5.28 gm of nitrogen sweat loss per day. If the above deduced nitrogen sweat losses for days 7 and 11 are used, and nitrogen balance is computed for these two days on the basis of the intake and urinary output figures given in table 3, then there is an apparent 3.37 and 3.58 gm negative nitrogen balance for days 7 and 11. Unquestionably these nitrogen losses in sweat are

TABLE 7

Diurnal and hourly sweat rate and nitrogen excretion in sweat when exposed to 100°F for 21 hours; mean excretion of three men per interval (1961 study)

Time	Day of exposure					
	7		11		15	
	Nitrogen	Sweat rate	Nitrogen	Sweat rate	Nitrogen	Sweat rate
hours[1]	*mg/hr*	*gm/hr*	*mg/hr*	*gm/hr*	*mg/hr*	*gm/hr*
0–3.5	158	362	198	363	—	293
4.5–8.5	245	438	216	399	238	376
9.5–11	202	430	207	399	178	376
11–15	240	430	204	436	194	334
15–23 (sleeping)	88	131	99	138	136	138
0–3.5 (next day)	255	412	224	413	261	413

[1] Zero time was 8 AM daily; no sweat collections between 3.5 to 4.5 hours, and 8.5 to 9.5 hours, which were meal times.

considerably higher than values of 0.5 and 0.8 gm/day quoted by Holmes et al. ('54) and Wallace ('59).

Rose and Wixom ('55) and Goyco ('59) in Puerto Rico, have reported nitrogen "equilibrium" with 3.5 and 4.6 gm of nitrogen/day, respectively, without considering the sweat losses. When one considers that the latter study was made in a hot tropical environment, the nitrogen losses in sweat must have been considerable.

In a review on the retention of dietary nitrogen, Patwardhan ('60) reported retentions of 1.0 to 5.0 gm of nitrogen with intakes of 1 to 1.6 gm of protein/kg body weight. These retentions were not accompanied by increases in body weight. Bray ('53) observed the same phenomenon in malnourished West African children, and Holmes et al. ('54) observed the same in malnourished African adults. Patwardhan ('60) feels that there is no satisfactory explanation for this phenomenon which without any weight gain, militates against the assumption underlying Rubner's calculation of 33 gm of new tissue for each gram of retained nitrogen by the body. Holmes et al. ('54) concluded that dermal nitrogen losses are too small (only 0.5 gm nitrogen/day) to introduce any serious errors and that these losses could not possibly account for the nitrogen retention over short periods, but could have greater effects over prolonged periods. If large quantities of nitrogenous material are lost by cutaneous structures, not only Holmes' study but many others will be invalidated.

Since these studies were all performed in hot, humid environments, a considerable quantity of the nitrogen retention could possibly be attributed to large nitrogen losses in sweat.

It has been speculated (Mitchell and Hamilton, '49) that the question of increased protein requirements depends upon the relationship between the dermal losses of nitrogen and the losses through the kidneys and alimentary tract. In the 1961 study the urinary and fecal nitrogen losses were remarkably constant signifying that the increased sweat nitrogen excretions are not compensated by decreases in the urine and feces. The possibility of increased protein requirements under conditions that produce profuse sweating should be reevaluated, especially in individuals living and working in extremely hot, dry or humid environments and who are consuming low protein diets. The sweat nitrogen losses of 13 to 14% of men consuming 80 to 90 gm of protein/day may not appear to be appreciable, but these excretions could be very significant for individuals consuming below 25 gm of protein a day (the minimal requirement) (FAO, '57). One should also remember that these values for nitrogen in sweat have been reported on the cell free sweat after centrifuging, so in actuality the total dermal losses may be even greater, due to the epithelial cells and other debris. Darke ('60) has estimated the debris to be approximately 20% of the total sweat nitrogen.

The data presented suggest that the daily protein requirements of men living and working in extreme heat, may need to be increased by at least 13 to 14% to compensate for the nitrogen losses in sweat but further studies at low protein levels will probably elucidate this problem.

SUMMARY AND CONCLUSIONS

The results of two experiments show that a considerable quantity of nitrogen is lost in sweat, under conditions that produce profuse sweating. These values average 149, 189 and 241 mg/hour during exposures to environmental temperatures of 70, 85 and 100°F, for men performing moderate daily physical activities.

Men performing a daily minimum of physical activity at 100°F showed a decreased excretion of from 300 to approximately 200 mg/hour, after acclimatization. It has been observed that the nitrogen losses in sweat increased with an increase in physical activity and sweat rate.

These observations are important since they show an additional nitrogen loss that has been ignored in many balance studies. Past studies where equilibrium was apparently attained should be reevaluated, with considerations for dermal nitrogen losses.

The increased nitrogen losses in sweat, even after acclimatization, are not compensated by decreased nitrogen losses from the kidneys and alimentary tract. As a result, the protein requirements of

0.35 gm/kg body weight, should be increased by at least 13 to 14% to compensate for the nitrogen losses in sweat.

The free amino acids excreted in sweat averaged more than 1 gm during a 7.5-hour exposure to 100°F temperatures. Data are also presented for other nitrogen compounds in sweat including urea nitrogen, ammonia, creatinine and uric acid.

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Excretion of Sodium, Potassium, Magnesium and Iron in Human Sweat and the Relation of Each to Balance and Requirements

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In two recent studies on calcium (Consolazio et al., '62a) and nitrogen (Consolazio et al., '62b) excretions in sweat, it was observed that sweat losses were fairly high, accounting for 18 to 30% of the total calcium excretion and 13 to 18% of the total nitrogen excreted, of men exposed to 100°F environmental temperatures. As a result of these fairly high excretions of calcium and nitrogen in sweat, other mineral contents were also investigated in relation to intake, balance and requirements. These minerals included sodium, potassium, magnesium, phosphorus and iron. In addition, the daily osmolarity was also computed from the freezing point of the sweat.

A considerable amount of data is available in the literature on the concentration of minerals in sweat, but very few, if any, of these studies have been related to balance and requirements. Many values for the sodium, potassium, magnesium, iron and phosphorous concentrations in sweat have been thoroughly reviewed by Schwartz ('60) and Altman and Dittmer ('61). Sodium values ranged from 13 to 104 mEq/liter of sweat (30 to 240 mg/100 ml), potassium from 2.3 to 16.0 mEq/liter (9 to 62 mg/100 ml), magnesium from 0.04 to 4.5 mg/100 ml, iron from 0.10 to 5.3 mg/hour, and phosphorus from 9 to 43 μg/100 ml of sweat.

The fairly high nitrogen and calcium excretions in sweat reported previously (Consolazio et al., '62a,b), especially during profuse sweating conditions, indicate that mineral losses in sweat may be sufficiently important to warrant further investigation. It is the opinion of investigators (Mitchell and Hamilton, '49; Mitchell and Edman, '62) that sweat losses should be included as part of the total daily output in balance studies, otherwise erroneous conclusions can be drawn.

An attempt was made to design a study to answer some of the following questions: (a) what are the mineral losses in relation to high sweat rates; (b) what are the mineral losses in relation to the daily intake and their effect on balance studies; (c) what are the mineral losses in relation to mineral allowances and requirements; and (d) do these sweat losses decrease after acclimatization?

EXPERIMENTAL DESIGN

A study was conducted for a total of 32 days on three healthy young men. It consisted on an 8-day preliminary period (1) at 24°C (75°F) and 50% relative humidity (RH), four 4-day periods at 37.8°C (100°F) and 70% RH (1, 3, 4 and 5), and an 8-day recovery or adjustment period at 24°C (75°F) and 70% RH (6), and sweat rates were computed daily for each man. Sweat samples were collected during morning and afternoon periods. Since the quantities of sweat collected during the control and adjustment periods at 75°F were limited, no values will be reported in this paper. The daily physical activity was constant and consisted of only 30 minutes of moderate activity on the bicycle (ergometer), the remainder of the day being spent in sedentary type activities. When the men left the test area (evening) no moderate to heavy activities were permitted, the test subjects being closely supervised by the Metabolic Ward personnel, during these hours.

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All of the test phases were performed in an environmental chamber from 8:00 AM to 11:30 AM and from 12:30 PM to 4:30 PM daily with the exception of each fourth evening when the men slept overnight in the hot room at 100°F. At this time sweat samples were collected for 2- to 5-hour intervals to determine whether the sweat concentrations of the minerals were decreased with longer exposure to the heat.

The menus consisted of 4 diets that were rotated during each period. The diets supplied 8.7 gm of sodium, 2.49 gm of potassium, 343 mg of magnesium, 23.4 mg of iron and 1.41 gm of phosphorus per day. All food was offered and consumed at regular mealtimes and no other food was permitted during the day. Salt was the only mineral available ad libitum and was measured for each man during each period.

Chemical analyses of the food composites, urine, feces and sweat were performed for sodium, potassium (Baird, '53), magnesium, iron, and phosphorus (Consolazio and Johnson, '60). In addition freezing points (Fiske, '54) were performed on the daily sweats.

Sweat rates were measured for each period using the method of Adolph et al. ('47). These sweat rates were determined by measuring weight changes during the morning and afternoon periods and then adjusting for water intake, and for water loss in urine and feces. (Gaseous exchanges of respiration contributes a small quantity, which usually consitutes less than 1% of the observed decrease in body weight.) Sweat samples were collected daily during both the morning and afternoon periods for a total of 7.5 hours. These samples were collected in polyethylene bags which covered one whole arm, and were representative for the entire period each subject was in the chamber. The arm and the bag were rinsed with distilled water and dried before each collection period began.

Complete urine and fecal collections were made for 4-day periods coinciding with the repetition of the 4 diets utilized. Mineral balances were computed on each man for each period, based on the inclusion and exclusion of the minerals in sweat as a source of mineral loss.

RESULTS

The chemical analyses of the various minerals performed on the cell free sweat are presented in quantities/100 ml of sweat (table 1). This table also includes the means and standard deviations during the 7.5 hours' exposure to 100°F temperatures. After the first 4 days of acclimatization to heat, the potassium excretion in sweat ranged from 25 to 28 mg/100 ml, magnesium excretion ranged from 0.61 to 0.64 mg/100 ml, iron from 25 to 39 μg/100 ml, and phosphorus from 0.11 to 0.26 mg/100 ml of sweat. The mean excretion rate in milligrams per hour for the same exposure periods ranged from 78–106 mg for potassium, 2.0–2.4 mg for magnesium, 0.11–0.16 mg iron, and 0.45–0.81 mg/hour for phosphorus. The sodium excretion in sweat was quite variable due to the fact that salt intake was not controlled (table 1). The daily 7.5-hour mineral excretion and total osmolarity in sweat for 16 consecutive days at 100°F are compared in table 2.

On three separate occasions when the men spent 24 hours continuously at 100°F, it was observed that the sweat rate and the sodium, potassium and iron excretions in

TABLE 1

Concentration of minerals in sweat;[1] mean for three men for 16 days of exposure at 100°F (1961 study)

Minerals	Days' exposure			
	1–4	5–8	9–12	13–16
Sodium, mg/100 ml	337 ± 183.0[2]	113 ± 67.5	118 ± 99.4	420 ± 296.3
Potassium, mg/100 ml	84 ± 72.1	25 ± 12.6	25 ± 6.3	28 ± 9.7
Magnesium, mg/100 ml	0.70 ± 0.29	0.61 ± 0.21	0.61 ± 0.16	0.64 ± 0.28
Iron, μg/100 ml	36.4 ± 21.2	33.2 ± 16.0	38.6 ± 21.1	25.0 ± 20.9
Phosphorus, mg/100 ml	0.155 ± 0.119	0.260 ± 0.152	0.109 ± 0.087	— —

[1] Concentration/100 ml of sweat as collected and centrifuged. Chemical analysis on supernatant fluid.
[2] Mean ± SD.

TABLE 2

Sweat excretion of minerals and total osmolarity; mean daily excretion of three men during daily 7.5-hour exposures at 100°F temperatures

Days of exposure 100°F	Sodium	Potassium	Magnesium	Iron	Phosphorus	Osmolarity[1]
	mg	*mg*	*mg*	*mg*	*mg*	*milliosmoles*
1	11176	2780	—	0.91	21.9	—
2	6210	3068	15.9	0.93	3.3	—
3	3535	658	18.5	1.13	3.8	194
4	—	—	—	—	—	221
5	2500	537	13.7	1.12	6.9	139
6	2379	555	15.4	0.81	5.4	131
7	—	—	—	1.12	—	143
8	4696	672	16.6	0.79	6.0	151
9	2623	660	17.3	0.76	3.1	112
10	3050	669	18.3	1.16	3.6	111
11	1644	516	18.6	1.26	5.2	111
12	4050	560	16.4	1.09	1.7	128
13	7090	772	17.5	0.83	—	120
14	7650	793	18.3	0.66	—	115
15	3380	757	18.6	1.41	—	135
16	6310	863	16.7	0.55	—	139

[1] −1.86°C for each 1000 milliosmoles.

TABLE 3

Diurnal variations of minerals in sweat, excretion per hour; mean for three men (1961 study)

Days at 100°F	Sodium			Potassium			Magnesium			Iron		
	7–8	11–12	14–15	7–8	11–12	14–15	7–8	11–12	14–15	7–8	11–12	14–15
hours[1]	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*μg*	*μg*	*μg*
0–3.5	375	234	944	57	74	108	3.2	2.3	2.8	159	107	179
4.5–8.5	844	289	1837	111	65	104	2.0	2.1	2.2	140	221	195
9.5–15	1235	1590	1331	115	101	82	2.2	1.8	1.5	110	155	84
15–23 (sleeping)	441	582	262	95	52	59	—	—	—	141	71	52
0–3.5 (next day)	626	945	841	90	85	115	1.8	2.2	2.2	101	193	73

[1] Zero time was 8 AM daily.

TABLE 4

Sodium balance, with and without sweat losses; mean for three men for 16 consecutive days (1961 study)

Days at 100°F	Intake	Output			Balance		Sweat as % of total output
		Urine	Feces	Sweat[1]	Sweat excluded	Sweat included	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	
1–4	10229	2600	105	5784	+7524	+1740	68.1
5–8	8729	2170	81	3188	+6478	+3290	58.5
9–12	8729	2360	95	2840	+6274	+3434	53.7
13–16	—[2]	2440	94	6110	—	—	—

[1] Includes only sodium excreted in sweat while in environmental chamber for 7.5-hour period. During three overnight exposures to 100°F the sodium excreted in sweat averaged 902 mg/hour or 14.88 gm/the 16.5-hour period.
[2] Extra salt from shaker not recorded.

sweat were decreased by approximately 50% during the sleeping hours. On arising and being active again, the morning sweat mineral losses were again at a higher level (table 3).

Mineral balances, during the 7.5-hour collection period, were computed for sodium, potassium, magnesium, iron and phosphorus. These balances were calculated with and without the inclusion of

TABLE 5

Potassium balance, with and without sweat losses; mean for three men for 16 consecutive days (1961 study)

Days at 100°F	Intake	Output			Balance		Sweat as % of total output
		Urine	Feces	Sweat[1]	Sweat excluded	Sweat included	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	
1–4	2493	2150	89	1776	+254	−1522	44.3
5–8	2493	2400	83	588	+10	−578	19.1
9–12	2493	1860	103	596	+530	−66	23.4
13–16	2493	1820	72	796	+601	−195	33.5

[1] Includes only potassium excreted in sweat for 7.5 hours in environmental chamber. During the three overnight exposures to 100°F, the potassium excreted in sweat averaged 84 mg/hour for a total of 1.39 gm for the 16.5-hour period.

TABLE 6

Magnesium balance, with and without sweat losses; mean for three men for 16 consecutive days (1961 study)

Days at 100°F	Intake	Output			Balance		Sweat as % of total output
		Urine	Feces	Sweat[1]	Sweat excluded	Sweat included	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	
1–4	343	25.7	107	17.2	+210.3	+193.1	11.5
5–8	343	23.6	112	15.2	+207.4	+192.2	10.1
9–12	343	23.1	102	17.7	+217.9	+200.2	12.5
13–16	343	21.9	76	17.8	+245.1	227.3	15.4

[1] Includes only magnesium excreted in sweat for 7.5 hours in environmental chamber. During the three overnight exposures at 100°F, the magnesium excreted in sweat averaged 1.82 mg/hour for a total of 29.7 mg during the 16.5-hour period.

TABLE 7

Iron balance, with and without sweat losses; mean for three men for 16 consecutive days (1961 study)

Days at 100°F	Intake	Output			Balance		Sweat as % of total output
		Urine[1]	Feces	Sweat[2]	Sweat excluded	Sweat included	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	*mg/day*	
1–4	23.4		20.5	1.01	+2.9	+1.9	4.7
5–8	23.4		19.4	0.96	+4.0	+3.0	4.7
9–12	23.4		21.1	1.07	+2.3	+1.2	4.8
13–16	23.4		21.5	0.86	+1.9	+1.0	3.9

[1] Negligible output.
[2] Includes only iron excreted in sweat for 7.5 hours in environmental chamber. During the three overnight exposures at 100°F, the iron excreted in sweat averaged 0.102 mg/hour, a total of 1.68 mg for the 16.5-hour exposure.

TABLE 8

Phosphorus balance, with and without sweat losses; mean for three men for 16 consecutive days (1961 study)

Days at 100°F	Intake	Output			Balance	Sweat as % of total output
	Food	Urine	Feces	Sweat		
	mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/day*	
1–4	1405	770	9.8	9.9	+625	1.2
5–8	1405	891	7.8	6.1	+500	0.7
9–12	1405	896	8.7	3.4	+497	0.4
13–16	1405	868	8.1	—	+529	—

TABLE 9

Total daily excretion of minerals; mean for three men (1961 study)

	Total excretion		7.5 hours	16.5 hours	Sweat excretion as % of total excretion	
	7.5 hours	16.5 hours[1]			7.5 hours	16.5 hours
	gm	*gm*	*mg/hour*	*mg/hour*		
Sodium	4.51	14.9	601	902	62.8	88.7
Potassium	0.94	1.39	125	84	30.1	52.0
	mg	*mg*				
Magnesium	17.0	29.7	2.3	1.8	12.4	27.5
Iron	0.98	1.68	0.131	0.102	4.5	11.0

[1] Minerals excreted in sweat during the regular 7.5-hour daily exposure and for three evenings when the men spent the remaining daily time (16.5 hours) at 100°F.

the sweat mineral losses. The sodium balance varied due to the variation in daily intake (table 4). The potassium balance was positive when the sweat losses were excluded but were negative when the sweat losses were included, averaging − 1522, − 578, − 66 and − 195 mg/day for the 4 consecutive 100°F periods (table 5). Magnesium balances did not vary greatly when comparing the inclusion and exclusion of the sweat losses, due to the extremely high retention of magnesium (table 6). Iron balances, even though they were reduced by approximately one-half, were still on the low positive side (table 7). The quantities of phosphorus in sweat were so small in comparison to the total daily intake that they did not change the highly positive balances significantly (table 8).

The sweat losses during the 7.5-hour exposure periods are presented as percentage of the total excretion in tables 4–8. Sodium in sweat accounted for 54 to 68%, potassium for 19 to 44%, magnesium for 10 to 15%, iron for 4 to 5% and phosphorus for 0.4 to 1.2% of the total excretion of each respective element. If one included an average value for mineral losses in sweat during the remaining 16.5 hours of the day when the men were exposed to 100°F temperature (days 7, 11 and 15) the total daily mineral excretion in sweat was approximately doubled (table 9).

DISCUSSION

It has been mentioned previously that sweat collections were made daily using the polyethylene bag, that covered one whole arm. The arm sweat which was representative for the entire period, was assumed to be comparable to the total body sweat. But a question arises whether this is a valid comparison. It has been shown by some investigators (Dill et al., '38; Johnston et al., '50; and Ladell, '48) that the various constituents of arm sweat are reasonably representative of the total body sweat. Van Heyninger and Weiner ('52) on the other hand are in disagreement, observing that the arm sweat is more concentrated than the rest of the body. Another factor to be considered is whether the arm bag will cause depression of the sweat rate. Collins and Weiner ('62) have observed a rapid depression of sweat gland activity in the forearm when the arm was covered with a sweat collection bag. Data are presented showing that the sweat rate is considerably less in humid environments than in dry conditions. These authors believe that the sweat depression in the arm bag may have been due to obstruction of the sweat gland ducts, by the excessive skin hydration.

Sodium is essential for the normal functioning of the body since it contributes to the acid-base balance of the body and since it is responsible in large measure, for the total osmotic pressure of the extracellular fluids (National Research Council, '58). Minimal allowances have not been established by the NRC due to lack of sufficient information, but it has been observed that a normal American intake will range from 3 to 7 gm of sodium/day. It has been reported (NRC, '58) that under normal conditions 90 to 95% of the

sodium intake is excreted in the urine and usually sweat sodium losses are not considered in sodium balance studies, under these conditions. Sodium can be excreted in sweat in fairly large quantities to disturb homeostasis and as a result seriously impair the economy of the organism (Schwartz, '60). Sodium excretion in sweat is variable, being dependent on physical activity, acclimatization, adrenal cortical activity, environmental temperature, humidity and body temperature. Under conditions of this study, where the daily sodium intake ranged from 8 to 10 gm/day, the losses of sodium in sweat accounted for more than 50% of the total daily excretion. Since the salt intake was ad libitum it was not possible to study the full effects of acclimatization on sodium excretion, but it was observed that the sweat sodium decreased appreciably during the first 12 days exposure to heat, which is in agreement with data reported in the literature. The sodium balance was highly positive by more than 3 gm even with the inclusion of the losses in sweat.

Potassium has been shown to be a required nutrient and is one of the principle basic elements in intracellular fluid, being found in greatest quantities within the cell. It is also a very important extracellular fluid constituent since it can influence muscular activity, notably the cardiac muscle, and can affect the excitability of the nerve tissue. Muscle protein repletions may demand an additional potassium intake of 2.7 mEq for each gram of nitrogen. The NRC ('58) has not established minimal allowances for potassium but a normal United States diet usually contains from 2.4 to 4.5 gm. An intake between 0.8 to 1.3 gm of potassium/day would be very close to the daily recommended allowances (NRC, '58). Potassium deficiencies, manifested by muscular weakness, increased nervous irritability, mental disorientation and cardiac irregularities, can be produced by gastrointestinal losses, by renal losses or by low potassium intake, and are frequently accompanied by metabolic alkalosis. The data in the literature are too limited to draw any specific conclusions on the relationship of potassium excretion in sweat and the total daily potassium excretion, as they may be related to such variables as body temperature, acclimatization to extreme heat, sweat rate and physical exercise. In some instances appreciable quantities of potassium in sweat have been reported (Schwartz, '60; Altman and Dittmer, '61) in man, living and exercising in extremely hot environments, but Davidson et al. ('59) and the British Ministry of Agriculture ('59) believe that sweat potassium losses are usually negligible, in relation to the daily intake. The potassium excreted in sweat accounted for approximately 30% of the total daily excretion. If the potassium losses in sweat for the remainder of the day were included, these losses could account for up to 50% of the total daily excretion. The potassium balances, with the addition of the sweat potassium losses, were all on the negative side.

Even though magnesium is a very important cellular constituent, there is very limited information in the literature in relation to magnesium metabolism in the body. It is known that for normal function, cardiac and skeletal muscles and nervous tissue depend greatly on a proper balance between calcium and magnesium ions. Magnesium will replace the calcium in bone salts when there is a calcium deficiency, but magnesium in excess will inhibit calcification. Even though the NRC has set up no minimal allowance, it has been calculated that the daily magnesium intake of adults in the United States is between 250 to 350 mg/day (NRC, '58).

Unlike sodium and potassium, the magnesium excretion in sweat did not decrease appreciably during acclimatization. The sweat accounted for approximately 12.5% of the total daily excretion of magnesium but when the overnight sweat losses are included, this excretion could account for 25% of the total. This sweat loss did not greatly affect the magnesium balance, since the daily retention was approximately 200 mg/day. This retention may be in part compensation for the negative calcium balance reported in the same study (Consolazio et al., '62a), or it may be that the estimate of the minimal daily allowances of 250 to 300 mg of magnesium/day may be too high. Under the conditions of this study, the magnesium loss in sweat is relatively unimportant, but in studies on

low magnesium intakes, these losses should be considered.

Even though a few studies have been performed on the relationship of sweat losses of iron to the daily requirements, the quantitative importance of the cutaneous iron losses is still a disputed issue. It is the feeling of one group (Foy and Kondi, '57) that the inclusion of the integumental iron losses may possibly increase the iron requirements by as much as 50% under non-stress conditions, and that these excessive sweat losses in hot environments may be a contributing factor in the development of iron deficient anemias. On the other hand Hussain and Patwardhan ('59) feel that the body tends to conserve iron in the anemia state by reducing the losses through the skin. Dubach et al. ('55) using radio iron as a tracer observed that during maximum sweating in normal humans, from 0.33 to 0.52 mg of iron may be lost in sweat in a 24-hour period.

Comparisons have been made on the cell-rich and cell-free sweat iron (Hussain and Patwardhan, '59) showing that the cell rich portion is from 3 to 10 times more concentrated than the cell free sweat. It was concluded that the major portion was present in the products of cellular desquamation; and since active thermal sweating is always accompanied by cell desquamation, the loss of iron in sweat could be of far greater importance than is generally supposed.

Iron loss via the sweat amounted to approximately 1 mg during the exposure period, which was 4.5% of the total daily excretion. If the iron losses in sweat during the remainder of the day are included, they could account for as much as 11% of the total daily excretion.

With a fairly high iron intake of 23.4 mg/day, the iron balance was positive by approximately 1 mg/day when the sweat iron losses were included. These sweat iron excretions are fairly high, especially if one includes the evening losses.

Prior to the beginning of the study the test subjects were on leave at home for a three-week interval. Hence, it is assumed that they were eating a normal diet which may have been comparable to the experimental diet. With the exception of potassium all of the mineral balances were positive. Under these conditions, the subjects could have been increasing body mass, but this may not be probable since the subjects lost a total of 1.05 kg during the experimental periods at 100°F (table 10).

TABLE 10

Body weight changes; mean change for three men for each 4-day period (1961 study)

	Days	Mean change in weight	
		kg/period	*kg/day*
Control period	1–4	+0.14	
	5–8	+0.49	
			+0.08
100°F period	1–4	+0.17	
	5–8	−0.21	
	9–12	−0.64	
	13–16	−0.37	
			−0.066
Recovery period	1–4	+0.77	
	5–8	−0.15	
			+0.08

It has been observed that sodium and potassium excretion in sweat decreased appreciably after acclimatization which is in agreement with data reported in the literature (Bass et al., '55; Dill et al., '38). On the other hand neither iron nor magnesium excretion in sweat decreased appreciably during acclimatization.

The data suggest that the excretion of sodium, potassium and iron in sweat are quite appreciable, especially under conditions that produce profuse sweating. These mineral losses must be considered in balance studies otherwise balance and daily minimal allowance data would be greatly misinterpreted. Studies in the literature where "equilibrium" was attained, under conditions of profuse sweating, should be reevaluated.

The concentration of phosphorus in sweat was found to be the lowest of any of the minerals analyzed in this study; averaging less than 0.5% of the total daily excretion. These values, which agree fairly well with data from other investigators, are relatively unimportant in computing balance studies.

The total osmolarity of sweat (freezing point) was decreased after acclimatization ranging from 116 to 141 milliosmoles after the acclimatization period. These values are comparable to other values for human sweat reported in the literature, and re-

viewed by Adams et al. ('58). These authors concluded that the osmotic concentration of sweat is hypotonic to serum and as the sweat increases, the osmotic concentration usually decreases.

SUMMARY

The results of this study show that a considerable quantity of sodium, potassium, magnesium and iron are lost in sweat of men during 16 consecutive days' exposure to environmental temperatures of 100°F. During a 7.5-hour collection period, the sweat excretions averaged 0.601 gm/hour for sodium, 0.125 gm/hour for potassium, 2.3 mg/hour for magnesium and 0.13 mg/hour for iron. Very small quantities of phosphorus are excreted in sweat, averaging between 0.45 to 0.81 mg/hour.

In the past, with the exception of sodium, very few investigators have recognized the fact that the mineral losses in sweat could be appreciable and as a result these losses have, all too frequently, been neglected in computing mineral balance. This could result in misinterpretation of the data, especially under conditions of profuse sweating. The total mineral loss should include the mineral loss in sweat. This in turn would help in estimating more realistically the minimal daily allowances of minerals.

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Essential Fatty Acid Deficiency and Rat Liver Homogenate Oxidations[1]

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It has been generally accepted that a deficiency of essential fatty acids (EFA) results in an elevated basal metabolic rate in the rat (Aaes-Jørgensen, '61). This has led to a series of investigations of the effect of EFA deficiency on various parameters of oxidative activity in cell-free liver preparations. Kunkel and Williams ('51) first noted an increase in cytochrome and choline oxidases but a marked decrease in endogenous respiration in essential fatty acid deficiency. However, Tulpule and Patwardhan ('52) reported that the deficiency resulted in a reduction of glutamic, succinic, and butyric acid dehydrogenase titers. Klein and Johnson ('54) first suggested that the deficiency brought about an uncoupling of oxidation from phosphorylation in mitochondria. This proposal was later supported by the work of Tulpule and Williams ('55) with homogenates and Levin et al. ('57) with mitochondria. The uncoupling of oxidation from phosphorylation is, therefore, believed to account for an increased oxidation of citric acid cycle intermediates by liver preparations from fat deficient rats.

Since the EFA's are major components of the phospholipids and lipoproteins, and thus of several subcellular structures, it appeared possible that many of the reported alterations in metabolic activity might be the result of altered subcellular structures. Levin et al. ('57) suggested that the uncoupling of oxidative phosphorylation in fat deficiency might be due to a greater susceptibility of mitochondria to structural alteration during their preparation. Increased mitochondrial swelling has also been reported in fat deficiency (Hayashida and Portman, '60). There is abundant evidence that fat deficiency results in a reduction in EFA concentration of liver subcellular fractions (Klein and Johnson, '54; Hayashida and Portman, '60). Thus far, however, data are lacking as to the course of development of the alteration in oxidative capacity of liver preparations and the role, if any, played by saturated fat in this development. Although a number of suggestions have been made, the exact nature of the oxidative changes has not been established nor is there universal agreement as to the oxidative systems affected.

It is the purpose of this paper to show that the oxidation of a wide range of substrates by liver homogenates is increased in EFA deficiency, whether the diet completely lacks fat or contains large amounts of saturated fat. This change appears as early as two weeks after feeding the deficient diet and is readily reversed within one week after feeding EFA. Present evidence suggests that the increased oxidation may be at least partially related to changes in mitochondrial structure.

METHODS

Weanling male albino rats of the Holtzman strain were housed individually in hanging wire cages and given food and water ad libitum. Weight data and observations of general physical appearance were recorded weekly. The rats were divided into 6 groups differing in the amounts and type of dietary fat they received. The EFA-deficient diets were either fat-free or included 25% of hydrogenated coconut oil.[2] Control diets for the former contained either 1.0% methyl

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[2] Hydrol, Durkee Famous Food Company, Chicago, Ill.

linoleate or 5% corn oil; for the latter they contained a combination of 22% of the hydrogenated coconut oil plus 3% methyl linoleate or 20% of hydrogenated coconut oil plus 5% corn oil. The other constituents common to all diets were alcohol-extracted casein (18% in low fat diets, 20% in high fat diets); L-cystine, 0.2%; calcium carbonate, 1.09%; equimolar phosphate, 0.9%;[3] fiber,[4] 3%; salts, 2% (Steenbock et al., '51); water-soluble vitamins 0.1% (DeLuca et al., '61); choline 0.2%; and glucose monohydrate[5] to give 100%. The fat-soluble vitamins were dissolved in ethanol and added to the diet containers to give 175 IU vitamin D_2, 1000 μg α-tocopherol, 120 μg 2-methyl-1, 4-naphthoquinone (menadione) and 80 μg vitamin A acetate/rat/week.

Gross symptoms of the EFA deficiency appeared by the eighth week in animals receiving 25% of the hydrogenated coconut oil and by the twelfth week in those receiving the fat-free diet. At this time, the rats were killed by stunning followed by decapitation. The livers were quickly removed and placed in ice-cold 0.25 M sucrose. A 10% homogenate in 0.25 M sucrose was made with a Potter-Elvehjem homogenizer fitted with a Teflon pestle and the rate of oxidation of a number of substrates was measured manometrically by standard Warburg techniques.

Subcellular particles were prepared by the differential centrifugation of a 10% homogenate in either 0.25 M sucrose or 0.44 M sucrose in an International refrigerated centrifuge, Model PR-2, using a slight modification of the technique of Schneider and Hogeboom ('50). The first fraction, which contained nuclei, unbroken cells, and cellular debris, was sedimented at 400 × *g* for 10 minutes and is referred to subsequently as the "debris fraction." The second, which sedimented at 8000 × *g* for 10 minutes and which contained mitochondria and heavy microsomes, is called the "mitochondrial fraction." Both fractions were washed once and suspended in sucrose of the appropriate concentration. The wash solution from the mitochondrial fraction was combined with the supernatant liquid remaining after isolation of mitochondria, and was termed the "supernatant fraction." The rate of oxidation of α-ketoglutarate was determined simultaneously for cell fractions, recombinations of cell fractions, and a sample of the original homogenate which was maintained on ice during the process of fractionation to insure equal aging. The nitrogen content of the homogenates and mitochondrial fractions was determined routinely by the method of Johnson ('41).

The incubation medium used in all oxidation studies contained 40 μmoles potassium phosphate buffer, pH 7.4; 6 μmoles adenosine triphosphate (ATP;) 0.08 μmoles cytochrome c (horse heart[6]); 20 μmoles magnesium chloride; 335 μmoles sucrose when 0.25 M sucrose was used as the suspending medium, and 468 μmoles sucrose when 0.44 M sucrose was used; substrates as given in table 1; and the 10% homogenate or the cellular fractions. Where required, the fractions were recombined to equal the concentration of particulate and supernatant fractions in the original homogenate. The total incubation volume in the flask was 3.0 ml; the center well contained 0.2 ml 10% KOH and the gas phase was air. The oxidations were followed for 30 minutes at 37°C for homogenates and at 30°C for the cell fractionation experiments.

To follow the development of the metabolic lesion, two rats from the group fed a fat-free diet and two from that receiving the 5% corn oil diet were killed at the intervals indicated in figure 1. The rate of oxidation of citrate by liver homogenates in 0.44 M sucrose was determined. A similar experiment was carried out using two groups of rats fed diets containing 25% of hydrogenated coconut oil, and 20% of hydrogenated coconut oil plus 5% corn oil, respectively. Four rats from the EFA-free group and two from the control group were killed weekly beginning on the second week and the homogenate oxidation of α-ketoglutarate was determined. At the end of the eighth week, some of the deficient rats were fed the control diet and the remaining deficient rats continued to be fed the EFA-free diet.

[3] Equimolar phosphate = KH_2PO_4 — K_2HPO_4 1:1.

[4] Cellu Flour, Chicago Dietetic Supply House, Chicago, Ill.

[5] Cerelose, Corn Products Company, Argo, Illinois.

[6] Sigma Chemical Company, St. Louis.

TABLE 1

Effect of dietary fat on the oxidative activity of liver homogenates[1]

Substrate[3]	No fat, 12 weeks	Dietary fat		
		5% Corn oil, 12 weeks	25% Saturated coconut oil, 8 weeks	25% Saturated coconut oil + 3% methyl linoleate, 8 weeks
	$QO_2(N)$[4]	$QO_2(N)$	$QO_2(N)$	$QO_2(N)$
Citrate	237 ± 9[5]	152 ± 9	236 ± 9	133 ± 9
α-Ketoglutarate	305 ± 16	204 ± 15	291 ± 12	160 ± 10
Glutamate	262 ± 13	191 ± 10	265 ± 8	168 ± 11
Succinate	391 ± 18	270 ± 11	362 ± 8	242 ± 12
Pyruvate[6]	276 ± 17	197 ± 12	297 ± 9	169 ± 8
Malate	226 ± 9	160 ± 11	218 ± 8	137 ± 6
β-Hydroxybutyrate	113 ± 12	122 ± 19	111 ± 16	120 ± 8
Caprylate[6]	312 ± 9	242 ± 15		

[1] Oxygen uptake was measured manometrically at 37°C for 30 minutes. Composition of the incubation medium is described in the text. Numbers in parentheses indicate number weeks the rats were fed the diet.
[2] At least 6 rats in each experimental group.
[3] All substrates were present at a concentration of 0.005 mmoles/ml except citrate (0.015 mmoles/ml), succinate (0.015 mmoles/ml), and caprylate (0.010 mmoles/ml).
[4] μl O_2/hr/mg N.
[5] SE of mean. The effect of EFA deficiency was significant ($P < 0.001$) with all diets and substrates except pyruvate ($P < 0.005$), caprylate ($P < 0.010$), and β-hydroxybutyrate (no significant difference). Each value represents an average of at least 6 determinations.
[6] 0.003 mmoles/ml fumarate or oxalacetate added.

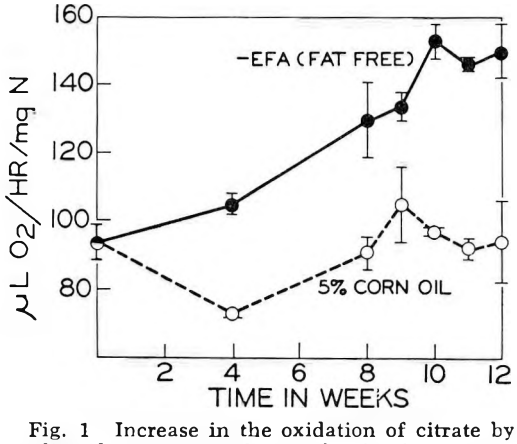

Fig. 1 Increase in the oxidation of citrate by rat liver homogenates during the development of EFA deficiency with a fat-free diet. Each point represents the average of two determinations and I represents the range. Experimental conditions are described in the text.

The experiment was then continued through the next 5 weeks.

RESULTS

Liver homogenates from rats deficient in EFA oxidized citrate, succinate, α-ketoglutarate, glutamate, malate, pyruvate, and caprylate, but not β-hydroxybutyrate at a higher rate than those from normal rats given EFA's (table 1). This effect was not altered by saturated fat in the diet, although the saturated fat greatly reduced the time of appearance of the lesion. Corn oil was virtually as effective as methyl linoleate in preventing the increase in oxidation with both high and low fat diets. To conserve space, therefore, only the data for either methyl linoleate or corn oil controls are presented.

For rats fed a fat-free diet, the oxidation of citrate was slightly elevated after 4 weeks and was appreciably increased by the eighth week (fig. 1). Maximal oxidation occurred two weeks later. In contrast, scaliness of the paws was present sporadically by the eighth week and consistently by the ninth. Tail lesions did not appear until the tenth or eleventh weeks. On the other hand, impairment of growth was evident as early as the fourth week of the deficiency and quite marked by the eighth week.

When a 25% hydrogenated coconut oil diet was fed (fig. 2), an increase in the rate of oxidation of α-ketoglutarate was noticeable by the second week. There was a steady increase through the first 5 weeks and a sharp increase in the eighth. In this case scaliness of the paws was observed in some animals as early as the fourth week, becoming universal by the fifth week. Tail lesions appeared three weeks later. Growth impairment was ob-

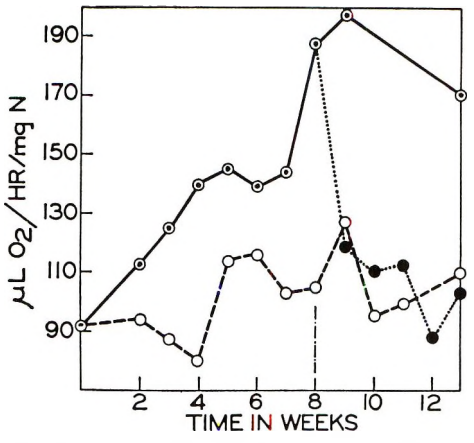

Fig. 2 Increased oxidation of α-ketoglutarate by liver homogenates during EFA deficiency induced by means of the 25% of hydrogenated coconut oil diet. Experimental conditions are described in the text. *Legend,* ⊙——⊙ = 25% hydrogenated coconut oil; ○- - - -○ = 20% hydrogenated coconut oil + 5% corn oil; ● . . . ● = deficient rats fed the control diet.

served by the second week, but the deficient animals did not stop growing during the 13 weeks of the experiment.

Feeding the deficient animals the control diet containing corn oil caused a marked decline in the rate of homogenate oxidation within one week. A sharp increase in growth rate was also observed within the same period but reversal of the dermal symptoms progressed more slowly. The rats appeared normal in all respects after 4 weeks.

Neither growth impairment in the absence of EFA's nor the resumption of growth observed in deficient rats given EFA's resulted in a change in food intake. Food consumption records taken during the eighth to eleventh weeks of the above experiment indicated that the rats consumed an average of 14 to 16 gm of food/day in both the deficient and EFA-fed groups. Although exact records of food consumption were not kept in the case of low fat diets, both deficient rats and those fed EFA's appeared to consume equal quantities of food. These results agree with those of others (Aaes-Jørgenson, '61) in which EFA deficiency causes a decrease in food utilization efficiency rather than in total food consumption. Therefore the effects observed in this study cannot be related to food intake.

In an effort to learn more about the subcellular site of EFA action on oxidative systems, homogenate fractionations were carried out in 0.25 M and 0.44 M sucrose. The oxidation rates of the different fractions from 0.44 M sucrose homogenates and their various combinations are shown in table 2. The data obtained with 0.25 M sucrose were similar to those shown in

TABLE 2

Oxidation of α-ketoglutarate by liver homogenate fractions from rats fed diets with and without EFA[1]

Cell fraction	No fat	Dietary fat		
		5% Corn oil	25% Hydrogenated coconut oil	22% Hydrogenated coconut oil + 3% methyl linoleate
	QO_2[2]	QO_2	QO_2	QO_2
Homogenate	4272 ± 354[3]	3147 ± 54	5010 ± 242	2690 ± 203
Debris	486 ± 96	117 ± 54	453 ± 86	71 ± 34
Mitochondria	1166 ± 138	1087 ± 150	1546 ± 169	874 ± 125
Mitochondria + debris	1703 ± 65	1304 ± 31	2196 ± 148	1183 ± 313
Mitochondria + supernatant	2554 ± 329	2850 ± 495	3244 ± 353	2133 ± 277
Debris + supernatant	860 ± 107	1150 ± 350	1163 ± 297	588 ± 212
Mitochondria + debris + supernatant	3260 ± 173	3000 ± 531	4075 ± 337	2415 ± 290

[1] Fractions isolated in 0.44 M sucrose. Incubations were followed for 30 minutes at 30°C. Incubation medium was as given in text.
[2] μl O_2/hr/gm tissue (wet weight).
[3] SE of mean. Each value represents an average of at least 4 determinations.

table 2 except that the separation of the mitochondria from the debris fraction was less complete.

The most striking effect of EFA deficiency was observed in the debris fraction. The mitochondrial fraction, nevertheless, showed an effect of deficiency, especially in the case of the high-fat diets. This effect was even more evident when the data were expressed on a mitochondrial nitrogen basis (table 3). The supernatant fraction in itself was incapable of α-ketoglutarate oxidation but, as expected, it stimulated oxidation carried out by the debris and mitochondrial fractions. Perhaps one of the more unexpected results was the poor recovery of oxidative capacity when the separated fractions from EFA-deficient liver were recombined in the same proportions as they existed in the original homogenates. In contrast, complete recovery was obtained routinely with homogenates from EFA-fed rats. The difference appears to lie in the greater ability of the supernatant fraction from EFA-fed rats to stimulate mitochondrial oxidation, since most of the activity in the normal homogenate was recovered by the combination of these two fractions. Because the homogenates were aged at 0°C to the same extent as the various fractions, these effects were not simply due to aging. However, this does suggest a greater lability of the EFA-deficient preparations to the fractionation manipulations. This might be expected if the EFA-deficient particles are more susceptible to structural damage as has been suggested (Levin et al., '57). In this respect, it is of interest that the 0.44 M sucrose isolation medium decreased the effect of EFA deficiency on mitochondrial oxidation (table 3) as it is well recognized that hypertonic sucrose concentrations have a protective effect on the structural integrity of mitochondria (Witter, '55).

DISCUSSION

The present results show clearly that EFA deficiency results in an increased oxidation of virtually all of the citric acid cycle intermediates as well as pyruvate and caprylate by liver homogenates. To our knowledge no systematic study of this type has been reported and existing data are conflicting. For example, Tulpule and Patwardhan ('52) reported a reduction in glutamic, succinic and butyric acid dehydrogenase activities of liver in EFA deficiency, whereas both Klein and Johnson ('54) and Tulpule and Williams ('55) noted the oxidation of succinate to be unaffected. In contrast, Levin et al. ('57) observed that succinate, as well as malate and α-ketoglutarate, was metabolized at a faster rate, but pyruvate oxidation was uninfluenced by EFA deficiency. It now appears that the metabolic defect occurs with virtually all the Krebs cycle intermediates, pyruvate, and caprylate.

Also important is that all previous studies of this type were carried out with rats fed a completely fat-free diet. The inclusion of saturated fat in the EFA-deficient diet has been reported to accelerate the appearance of the dermal symptoms and to intensify them in proportion to the amount of fat fed (Peifer and Holman, '59). The appearance of the metabolic defect of liver homogenates apparently is accelerated but not intensified by the addition of saturated fat to the EFA-deficient

TABLE 3

Mitochondrial oxidation of α-ketoglutarate[1]

Dietary fat	Isolation medium	
	0.25 M Sucrose	0.44 M Sucrose
	$QO_2(N)$[2]	$QO_2(N)$
No fat	298 ± 25[3]	362 ± 16
5% Corn oil	174 ± 15	257 ± 21
25% Hydrogenated coconut oil	228 ± 22	337 ± 33
20% Hydrogenated coconut oil + 5% corn oil	129 ± 16	237 ± 20

[1] Oxygen uptake was measured manometrically at 30°C for 30 minutes. Composition of the incubation medium is described in the text.
[2] μl O_2/hr/mg N.
[3] SE of mean. Each value represents an average of at least 6 determinations.

diet. Further, the results indicate that the effect of fat deficiency on oxidative activity was due to lack of EFA per se rather than of dietary fat.

The development of an increased liver homogenate oxidation is closely related to the overall course of development of EFA deficiency. The original metabolic studies of Watson and Burr ('31) indicated a high metabolic rate, especially in rats just entering the condition recognizable as characteristic of the deficiency disease. Later Panos and Finerty ('54) pointed out that increased oxygen consumption is perhaps the earliest manifestation of fat deficiency, a difference being evident as early as the second week. Klein and Johnson ('54) noted that during the first 6 weeks marked changes occur in the polyunsaturated acid content of subcellular particles in rats fed a fat-free diet. Thereafter, the losses proceeded very slowly, if at all. These changes, therefore, appear to be some of the earliest recognizable symptoms of the deficiency. On the basis of the data presented here, it appears that an increased liver homogenate oxidation should be included among the early changes occurring in EFA deficiency. The appearance of this lesion seems to correlate rather well with the changes in polyunsaturated acids observed by Klein and Johnson ('54) and with repression of growth. It has been repeatedly shown that the EFA's are present in high concentrations in the phospholipids involved in cellular structure (Richardson et al., '61; Marco et al., '61). Further, the mitochondria have been shown to have a relatively short half-life of approximately 10 days (Fletcher and Sanadi, '61). This may explain, perhaps, the early appearance of the lesion as well as its rapid reversal by EFA's. However, although it is reasonable that changes in cellular or subcellular structure would influence metabolism, possibly by way of increased permeability, neither the changes in polyunsaturated acids nor the increase in the oxidative activity of the liver occur early enough and to a sufficient degree to account for the early increased oxygen consumption reported by Panos and Finerty ('54).

Fractionation of homogenates revealed that the major effect of EFA deficiency is found in the debris fraction. If it can be assumed that cytochrome oxidase, an enzyme necessary for the oxidation of citric acid cycle intermediates, is localized solely in the mitochondria as is generally accepted (Schneider, '46), the effect occurring in the debris fraction could be due in part to greater mitochondrial contamination of that fraction. Such an abnormal sedimentation pattern would result from either the more rapid sedimentation of dense particles or the mechanical entrapment of large, swollen mitochondria by the nuclei and debris. The existence of enlarged liver mitochondria in EFA deficiency has been reported (Levin et al., '57) and unpublished results obtained in this laboratory indicate that deficient mitochondria may be two or three times larger than normal mitochondria. Alternatively, if the sedimentation pattern is normal, then the mitochondria left in the debris fraction must be altered in such a way that they have a much greater oxidative activity than the recovered mitochondria. This, in turn, would require that the mitochondria isolated in the appropriate fraction could not be representative of the total population of mitochondria. That an effect of EFA deficiency can be demonstrated in isolated mitochondria (table 3) tends, however, to favor the former possibility. The present results would therefore be in harmony with the previous suggestions of Levin et al. ('57) and of Hayashida ('60) that liver mitochondria from EFA-deficient rats are morphologically altered and tend to swell during their isolation.

SUMMARY

Essential fatty acid deficiency (EFA) in rats, whether induced by a diet containing no fat or a large quantity of saturated fat, results in an increased oxidation of practically all of the citric acid cycle intermediates, pyruvate, and caprylate by liver homogenates. This increase was detectable after rats are fed a high fat diet for two weeks, and a fat-free diet for four weeks. These elevated oxidative rates were reduced to the normal range within one week after feeding a source of EFA.

Fractionation of liver homogenates revealed that the greatest effect of EFA

deficiency is associated with the debris fraction. However, the isolated mitochondria from deficient rats, nevertheless, showed an increased oxidation rate when the results are expressed on an equivalent nitrogen basis.

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Metabolic Patterns in Preadolescent Children

VII. INTAKE OF NIACIN AND TRYPTOPHAN AND EXCRETION OF NIACIN OR TRYPTOPHAN METABOLITES[1]

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There is little information on the urinary excretion of niacin[2] and tryptophan derivatives by normal preadolescent children. This paper brings together the results obtained on the quantitative excretion of several niacin metabolites from metabolic studies with children maintained with relatively constant diets of known composition. The data will outline the excretion patterns of 7- to 9-year-old girls and indicate trends and factors affecting niacin metabolism in children.

Studies reported on adults have shown a relationship between niacin requirement and caloric intake (Goldsmith, '56; Horwitt and associates, '56); Goldsmith ('56) also reported niacin requirement to be related to body weight. Studies on urinary excretion of niacin and tryptophan metabolites by adults indicate that approximately 60 mg of dietary tryptophan are equivalent to 1 mg of niacin (Horwitt et al., '56),[3] a concept summarized by Goldsmith ('58) and Horwitt ('58).

PLAN OF METABOLIC STUDIES AND EXPERIMENTAL PROCEDURE

A series of three metabolic studies was carried out with 35 healthy girls 7 to 9 years of age. The first study ('54) was conducted in the fall of the year while the girls were attending school and the two subsequent studies ('56 and '58) were made during the summer.

The controlled diets for these studies were made up of ordinary foods and, except for protein, provided nutrients at levels recommended for this age group (NRC, '53). In 1954 the 11 girls were maintained with *diet 1* which furnished daily about 2 gm of protein/kg of body weight, the recommended level for 7- to 9-year-old children. In 1956 the 12 girls were divided into two groups on the basis of body weight, 6 small and 6 large girls. Half of each group received about 2 gm of protein/kg of body weight (*diets 2 and 4*) and the other half about 3 gm/kg (*diets 3 and 5*). To bring the protein content of the '56 diets to the desired levels, diets 3, 4, and 5 contained daily 3, 4, and 19 gm, respectively, of gelatin, hydrolyzed with fresh pineapple juice or papain. The 1958 study with 12 girls was carried out to determine the metabolic patterns when the dietary protein was about 0.8 gm/kg of body weight (*diet 8*) and when reduced further to about 0.6 gm/kg (*diet 8'*). A detailed description of each study and information on the kinds and amounts of foods in each diet, together with data on the physical and biochemical status of each subject are presented in Southern Cooperative Series Bulletin no. 64 ('59).

In the two studies when dietary protein was at or above the recommended level, the niacin was furnished mainly from ordinary food sources. But in 1958, when

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[2] Niacin denotes the vitamin whether it is in the amide or free acid form.

[3] Goldsmith, G. A., O. N. Miller and W. G. Unglaub 1956 Efficiency of tryptophan as a niacin precursor. Federation Proc., 15: 553 (abstract).

the protein was below the recommended level, the niacin supplied by ordinary foods was supplemented with niacinamide given in capsules and niacinamide added to the bread dough.

Analyses were made on food composites and on urine composites collected on alternate 4-day periods throughout the 64-day metabolic study in 1954, the 56-day study in 1956, and on all 6-day periods for the 48-day study in 1958.

All urine was collected for 24-hour periods, kept under refrigeration during each collection period, and held in the frozen state until pooled into a composite specimen for each metabolic period. A portion of each pooled specimen was neutralized (pH 6.9 ± 0.1) and frozen until analyzed for tryptophan and niacin metabolites. Analyses for nicotinic acid, N^1-methylnicotinamide, quinolinic acid, and tryptophan were carried out with methods previously described (Goldsmith et al., '52) and for N^1-methyl-2-pyridone-5-carboxamide with the method of Price ('54).

The food composites were analyzed microbiologically for niacin (Association of Vitamin Chemists, '51) and chemically for tryptophan (Horn and Jones, '45).

RESULTS AND DISCUSSION

The average weight of the group of girls supplied with each of 7 diets and the average daily nitrogen, energy, niacin, and tryptophan content of each diet are shown in table 1. The average daily amount of niacin furnished by the 7 diets ranged from 7.7 to 12.5 mg and dietary tryptophan from 141 to 882 mg; in general both paralleled the range for dietary nitrogen, except in diets 8 and 8′. Diet 1 was planned to furnish nutrients at levels recommended by the National Research Council ('53) for children 7 to 9 years of age and provided daily an average of 10.4 mg of niacin and 767 mg of tryptophan.

The prestudy diets of the girls calculated from records of their self-selected food intake for two weeks indicated that the average daily niacin intake of the girls studied in 1954, 1956, and 1958 was 12.7, 13.5, and 11.8 mg, respectively (Southern Cooperative Series Bulletin no. 64, '59). These calculated niacin intakes did not include an estimate of the niacin formed from dietary tryptophan.

In table 2 the combined niacin and tryptophan content of each diet is expressed as niacin-equivalents (NE), thereby making allowances for the niacin compounds which could be derived from tryptophan at the average conversion reported for adults. Also presented in table 2 are data on average urinary excretions of the following niacin-related consituents: N^1-methylnicotinamide (N^1-Me), N^1-methyl-2-pyridone-5-carboxamide (pyridone), nicotinic acid, tryptophan, and quinolinic acid. The sum of the average daily excretion of N^1-Me and pyridone, both expressed in terms of nicotinic acid, plus the nicotinic acid, is shown as a combined value for niacin metabolites (NM) for each group of subjects.

TABLE 1

Average weight of girls and daily nitrogen, energy, niacin, and tryptophan content of the diets

Year of study	Diet no.[1]	Subjects		Average daily intake			
		No.[1]	Wt[2]	Nitrogen	Energy	Niacin	Tryptophan
			kg	*gm*	*Cal.*	*mg*	*mg*
1954	1	1–11	29.5	10.4	1955	10.4	767
1956	2	12–14(S)	24.0	7.7	1948	7.7	555
	4	18–20(L)	30.8	9.4	2356	9.2	667
	3	15–17(S)	27.0	11.6	1966	9.9	853
	5	21–23(L)	32.3	14.1	2415	10.7	882
1958	8	24–35	27.5	3.5	2177	12.5	189
	8′	(same)	28.4	2.9	2240	12.2	141

[1] Diet and subject numbers correspond with those in Southern Cooperative Series Bulletin no. 64 ('59). The 1956 diets were adjusted for small (S) and large (L) girls.
[2] Average initial body weight of each group of girls.

TABLE 2

Intakes of niacin equivalents (NE) for 35 girls, 7 to 9 years of age, and urinary excretions of N¹-methylnicotinamide (N¹-Me), pyridone, nicotinic acid, tryptophan, and quinolinic acid

Subject no.[1]	Average daily intake			Average daily urinary excretion							
	NE[2]	NE/1000 Cal.	NE/kg body wt	N¹-Me	Pyridone	Nicotinic acid	Combined NM[3]			Tryptophan	Quinolinic acid
							Total	Range	% of NE		
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*		*mg*	*mg*
1–11	23.2	11.9	0.79	3.2	10.5	0.29	11.7	7.2–17.7	50	9	5.3
12–14	16.9	8.7	0.70	2.6	4.2	0.17	5.9	2.1–12.1	35	5	4.4
18–20	20.3	8.6	0.65	2.6	5.9	0.26	7.4	3.5–13.2	37	6	5.7
15–17	24.2	12.3	0.89	3.0	8.4	0.21	9.7	4.5–16.2	40	6	3.6
21–23	25.4	10.5	0.78	2.3	6.6	0.31	7.7	4.5–11.0	31	8	3.0
24–35	15.6	7.2	0.57	2.5	5.2	0.28	6.8	3.6–11.2	43	8	3.2
(same)	14.5	6.8	0.51	2.9	5.3	0.29	7.2	5.1– 8.8	50	6	3.2

[1] Subject numbers correspond with those in Southern Cooperative Series Bulletin no. 64 ('59).
[2] Niacin equivalent indicates dietary niacin plus 1/60 dietary tryptophan.
[3] Combined niacin metabolite (NM) excretion indicates N¹-Me plus pyridone (both expressed in terms of nicotinic acid) plus nicotinic acid. The range includes the average daily NM excretion for every metabolic period.

Urinary excretion of NM in relation to NE intake. A summary of regression analyses of urinary excretion of combined NM on NE intake is given in table 3. The relationship of outgo to intake was significant when the NE intake was expressed in milligrams per day, milligrams per 1000 Cal., or milligrams per kilogram of body weight and regardless of whether data for the girls consuming gelatin-containing diets were included or excluded. In figure 1 are shown the data, according to subjects, for urinary excretion of NM plotted against the intake of NE expressed in milligrams per day and in milligrams per 1000 Cal. The data for girls fed the diets with and without gelatin are identified separately. Regression lines are given for all subjects and for the subjects fed diets without gelatin.

With similar NE intakes, NM excretions for the girls fed diets with gelatin tended to be lower than excretions for the girls consuming diets without gelatin (fig. 1). These results suggest that with the three diets containing gelatin, which furnished daily 20.3, 24.2, and 25.4 mg of NE and 59, 73, and 88 gm of total

TABLE 3

Regression of urinary excretion of niacin-tryptophan metabolites (NM) on niacin equivalent intake (NE) for girls 7 to 9 years of age

Regression	No. of subjects	Regression coefficient[1]	Proportion of variation explained by NE intake[2]
			%
NE intake, mg/day, on NM excretion, mg/day			
Including diets with gelatin	47	0.39	47
Excluding diets with gelatin	38	0.57	72
NE intake, mg/1000 Cal., on NM excretion, mg/day			
Including diets with gelatin	47	0.78	56
Excluding diets with gelatin	38	0.92	66
NE intake, mg/kg body weight, on NM excretion, mg/day			
Including diets with gelatin	47	7.40	21
Excluding diets with gelatin	38	8.98	25

[1] Change in excretion for an increased intake on NE of one unit (mg/day, mg/1000 Cal., or mg/kg). All were significant at 1% level.
[2] Square of correlation coefficient.

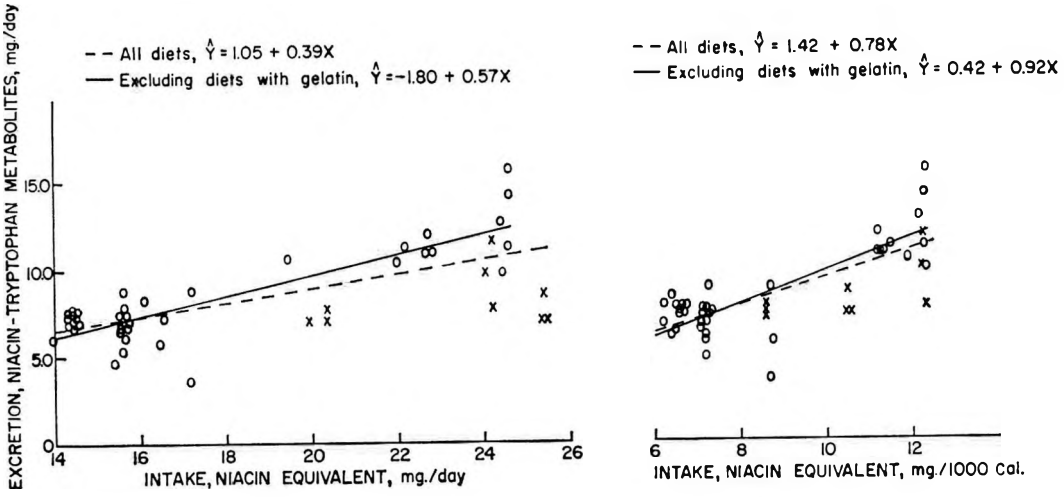

Fig. 1 Urinary excretion of niacin-tryptophan metabolites (N^1-methylnicotinamide plus pyridone, both expressed in terms of nicotinic acid, plus nicotinic acid) in relation to niacin equivalent intake expressed as: milligrams per day and milligrams per 1000 Cal. The symbol (o) refers to the average excretion for each girl (subjects 1–14, 24–35) using diets without gelatin and the symbol (x) to the average excretion for each girl (subjects 15–23) receiving diets containing gelatin.

protein (N × 6.25), respectively, the conversion of tryptophan to niacin was less than with diets without gelatin. Furthermore, the retention of nitrogen was less than for the girls fed the gelatin-containing diets, even though these diets were relatively high in protein. In reporting these results Speirs and associates ('60) suggested that these lower values may have been caused by an amino acid imbalance.

It is uncertain whether substances causing amino acid imbalance act by affecting utilization of tryptophan or niacin or by affecting the formation of niacin from tryptophan. Amino acid imbalance, however, appears to increase niacin requirement. Observations in this study suggest that niacin was used to spare tryptophan and that there was some inhibition of conversion of tryptophan to niacin. A preferential use of tryptophan has been shown by Vivian et al. ('58) who used diets low in niacin and tryptophan supplemented with gradually increasing levels of tryptophan. They found that tryptophan was used first to maintain nitrogen balance, then for synthesis of pyridine nucleotides, only after this was there increase in urinary excretion of niacin metabolites.

The average percentage of the NE intake excreted, expressed as combined NM (table 2), shows that there was considerable variation in average daily excretion within groups of girls receiving relatively constant intakes of NE. It is possible that the body niacin or tryptophan stores of some of the girls were greater than those of others. For example, the average excretion of 35% of the NE intake for the three girls fed the only diet without gelatin used in 1956 (diet 2) was unduly influenced by one subject whose excretion of NM accounted for only 21% of the intake, the lowest average excretion found among the 35 subjects. The average for the other two girls consuming diet 2 was 42%. The girls receiving diets with added gelatin (diets 3, 4, and 5) with average daily NE intakes ranging from 20.3 to 25.4 mg excreted from 31 to 40% of the intake as NM. The girls given diets without gelatin (diets 1, 2, 8, and 8′) with NE intakes ranging from 14.5 to 23.2 mg, excreted from 35 to 50% of the NE intake or from 42 to 50% when the one extreme case is excluded.

Urinary excretion of N^1-Me and pyridone. In figure 2 are shown the range and average daily urinary excretion pattern for N^1-Me and pyridone for the group

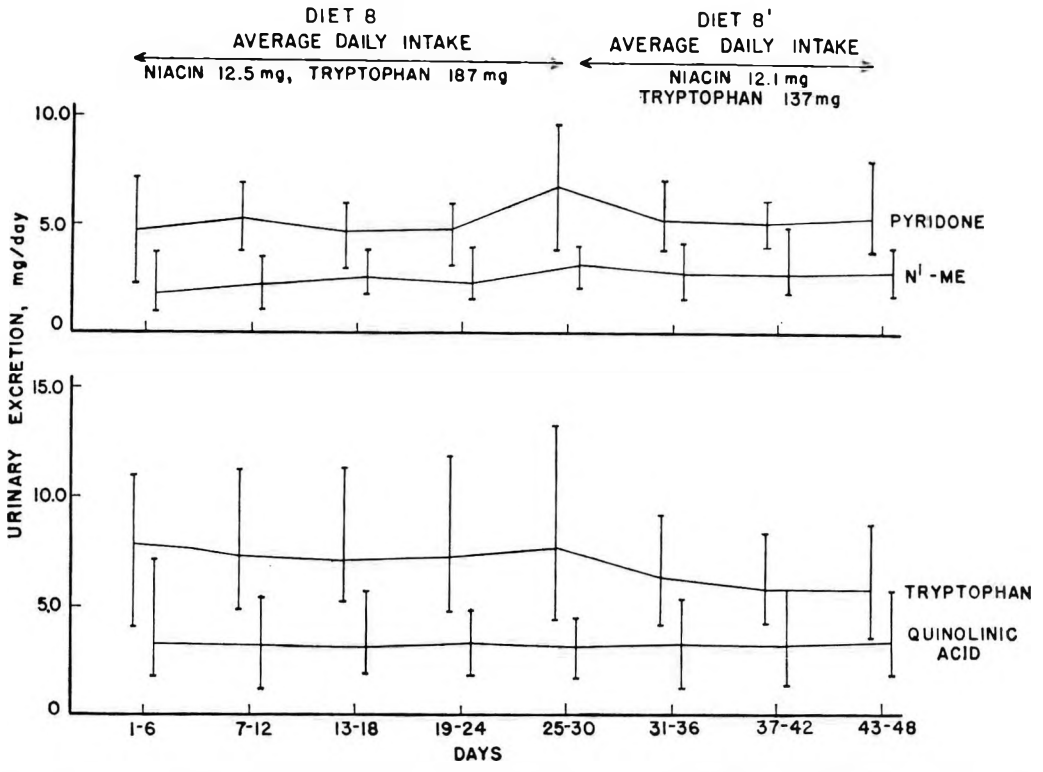

Fig. 2 Urinary excretion of pyridone, N¹-methylnicotinamide (N¹-Me), tryptophan, and quinolinic acid in successive 6-day metabolic periods with low protein diets. Each vertical line represents the range for 11 girls (subjects 24–34), the horizontal line connects the average excretions for the 8 metabolic periods.

of girls in successive 6-day metabolic periods receiving two low levels of protein; namely, 0.8 gm/kg of body weight (diet 8) and 0.6 gm/kg (diet 8′). Diet 8′ was the same as diet 8 except that 120 ml of milk was omitted from the daily food intake. The average excretion of N¹-Me remained constant as the study progressed but the pyridone increased for days 25 to 30 (period 5). There is no explanation for the increased excretion of pyridone in period 5 other than that the average daily niacin intake was about 1 mg higher than in the other metabolic periods.

The average ratio of urinary pyridone to N¹-Me for the girls ('54) having an average NE intake of 23.2 mg/day was 3.4. With a comparable intake ('56), when 9 of 12 girls received diets with added gelatin, the average ratio dropped to 2.7. When the NE intake was 15.6 mg/day and with further reduction to 14.5 ('58), the respective ratios of the pyridone to N¹-Me excreted were 2.2 and 2.0. The results of these studies indicate that the ratio of pyridone to N¹-Me in the urine tended to decline as the dietary NE declined.

In experimental pellagra Goldsmith and associates ('56) found that at low levels of excretion about equal amounts of pyridone and N¹-Me were found in the urine. In a study with college women consuming a diet containing daily 680 mg of tryptophan and 10 mg of niacin, Vivian et al. ('58) reported an average pyridone-to-N¹-Me ratio of 1.42 when excretions were calculated on the molar basis. When the daily tryptophan intake was lowered to 170 mg and niacin to 2.5 mg, the mean pyridone-to-N¹-Me ratio declined to 0.52.

Rosenthal et al. ('53) suggested that niacin intake in excess of requirement for normal metabolism is disposed of as the pyridone. If this is true, the pyridone-to-N¹-Me ratios of 2.0 and above noted in

urines for 7- to 9-year-old girls would indicate that the niacin requirements appeared to have been fulfilled in these subjects when the NE intake was around 14 mg/day, the recommended allowance (NRC, '58).

Urinary excretion of nicotinic acid. The excretion of niacin as measured microbiologically, averaged about 0.2 to 0.3 mg daily and accounted for only a small amount of the total NM excreted (table 2). The excretion appeared to be independent of the intake of niacin, tryptophan, or the NE of the diet. The nutritional significance of niacin excretion remains to be elucidated.

Studies with adults have indicated that niacin excretion remained unchanged even though the subjects developed severe niacin deficiency (Goldsmith et al., '52). Horwitt et al. ('56) stated that niacin in urine is not directly related to the nutritional state of the individual.

Urinary excretion of tryptophan. In figure 2 are shown the range and the average daily excretion of tryptophan for successive metabolic periods for 11 girls receiving the low-protein diets (8 and 8′). Data for subject 35 are excluded because she received milk with diet 8′. As the protein intake declined from 0.8 to 0.6 gm/kg of body weight and the tryptophan from 187 to 137 mg, the average amounts of tryptophan excreted declined from around 8 to 6 mg and the range in excretion narrowed; however, the reduction in tryptophan excretion was not significant. In table 2 are shown data on the average daily urinary excretions of tryptophan for the girls maintained with each of the 7 diets. The data show that the average excretion of tryptophan appeared to be about the same for all groups.

Urinary excretion of quinolinic acid. Results obtained for subjects receiving the low-protein diets shown in figure 2 indicate that the average daily excretion of quinolinic acid, a metabolic derivative of tryptophan, was fairly constant at about 3 mg daily and was not affected by lowering the daily dietary tryptophan from 187 mg to 137 mg. The data in table 2 show that on tryptophan intakes ranging from 555 to 853 mg daily (diets 1 to 4), the average quinolinic acid excretion was about 4 to 6 mg/day. These data suggest that the girls having the higher tryptophan intakes tended to excrete larger amounts of quinolinic acid. An exception to this were the results for the girls consuming diet 5. This diet contained the largest amount of tryptophan and the largest amount of gelatin (which is almost devoid of tryptophan) and resulted in the lowest excretion of quinolinic acid, an average of 3 mg daily. When gelatin provided as much as 22% of the dietary protein it may have depressed the formation of quinolinic acid from tryptophan.

Dietary niacin-tryptophan relationship. It is not known whether the tryptophan needs for protein synthesis in the growing organism take priority over the conversion of the amino acid to niacin. In studies with college women it appeared that tryptophan was used first to establish and maintain nitrogen equilibrium (Vivian et al., '58).

There was considerable variation in the average nitrogen retentions among the girls (James, '60) and in the average retentions among the groups of girls eating the different diets (Coons and Moyer, '60). With diets providing 7.7 to 11.6 gm of nitrogen and 555 to 853 mg of tryptophan daily (*diets 1 to 4*), the girls retained an average of 41 to 51 mg of nitrogen/kg of body weight. With somewhat higher nitrogen and tryptophan intakes, but with 19 gm of the dietary protein given as hydrolyzed gelatin (*diet* 5), the nitrogen retention was 35 mg/kg. With the diet providing 3.5 gm of nitrogen and 189 mg of tryptophan (*diet* 8), the average nitrogen retention was 24 mg/kg and with lower intakes of 2.9 gm of nitrogen and 141 mg of tryptophan (*diet* 8′), the nitrogen retention decreased to 15 mg/kg of body weight. It appears likely that only small amounts of tryptophan were available for conversion to niacin for the girls maintained with diets 8 and 8′.

In studies with adults eating diets that furnished 200 mg of tryptophan and from 4.6 to 21.2 mg of niacin daily, Goldsmith ('56) reported that the combined excretion of N^1-Me and pyridone was 0.2 mg for each milligram of niacin ingested when the intake was less than 8 to 10 mg daily, and 0.6 mg for each milligram of niacin in-

gested when the intake was above this level.

The combined excretion of NM for each milligram of NE or niacin ingested by these preadolescent girls was calculated, assuming that dietary tryptophan in excess of 200 mg was available for conversion to niacin compounds for the girls receiving diets 1 to 5, and that none of the tryptophan was available for conversion with diets 8 and 8′. On the basis of this calculation, the combined excretion of NM for each milligram of NE ingested was 0.6, 0.4, 0.4, 0.5, and 0.4 mg with NE intakes of 19.8, 13.6, 17.0, 20.8, and 22.1 mg daily, respectively, (diets 1 to 5) and 0.5 and 0.6 mg for each milligram of niacin ingested with niacin intakes of 12.5 and 12.2 mg daily, respectively, (diets 8 and 8′).

SUMMARY

Three metabolic studies were carried out with a total of 35 normal 7- to 9-year-old girls maintained with controlled diets varying in niacin and tryptophan as well as protein content. Determinations were made for dietary content of niacin and tryptophan.

Urinary excretions were determined for nicotinic acid, N^1-methylnicotinamide (N^1-Me), N^1-methyl-2-pyridone-5-carboxamide (pyridone), quinolinic acid, and tryptophan. Intake was expressed as niacin equivalent (NE) using niacin plus 1/60 tryptophan. Outgo was expressed as niacin metabolites (NM) using the combined excretion of N^1-Me, pyridone, and nicotinic acid, all expressed as nicotinic acid.

The relationship between outgo of NM and intake of NE was positive and linear when average NE intakes varied from 14.5 to 25.4 mg/day, from 6.8 to 12.3 mg/1000 Cal., or from 0.51 to 0.89 mg/kg of body weight.

For the 26 girls using diets without gelatin and average daily NE intakes ranging from 14.5 to 23.2 mg, the average daily NM excreted ranged from 42 to 50% of the intake, with exception of 21% for one girl. For the 9 girls fed diets with added gelatin and average daily NE intakes ranging from 20.3 to 25.4 mg, the average daily NM excreted ranged from 31 to 40% of the intake. The lower excretion of NM for the girls eating the gelatin-containing diets suggests that less tryptophan was converted to niacin. This may have been the result of an amino acid imbalance.

The average ratio of pyridone-to-N^1-Me in the urine decreased from 3.4 to 2.0 as the average dietary NE decreased from 23.2 to 14.5 mg/day.

The excretion of nicotinic acid averaged about 0.2 to 0.3 mg daily. The average excretions of tryptophan ranged from 5 to 9 mg daily, but for one group of 11 girls, excretions decreased when the daily protein intake was reduced from about 0.8 to 0.6 gm/kg of body weight and the tryptophan from 187 to 137 mg. Quinolinic acid tended to be excreted in larger amounts by the girls eating higher protein diets furnishing from 555 to 853 mg of tryptophan daily, and to be depressed for the girls receiving the diet containing an average of 882 mg of tryptophan and 19 gm of gelatin daily.

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Influence of Lactose and Dried Skim Milk upon the Magnesium Deficiency Syndrome in the Dog

I. GROWTH AND BIOCHEMICAL DATA[1]

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The increased absorption of calcium, phosphorus and strontium as a result of the inclusion of lactose in the diet has been clearly demonstrated. The effect of dietary lactose on magnesium absorption and utilization has been studied less extensively. Outhouse et al. ('38) observed that the inclusion of lactose in the diet of rats resulted in a greater retention of magnesium. Forbes ('61), however, found that the slight increase in the absorption of magnesium as a result of lactose in the diet was more than offset by the increased urinary excretion. Heggeness ('59) observed that an increased urinary excretion of magnesium also resulted when the diet contained 60% of galactose. Lengemann ('59) found that a dosing of lactose (1 mmole) with Mg^{28} resulted in an increased percentage of the dose of Mg^{28} deposited in the femur of rats.

Dreizen et al. ('61) reported smooth muscle degeneration and necrosis in the media of the aorta and in the muscular wall of the stomach of 6-week-old rats restricted to a diet of nonfat dry cow's milk and tap water. Rats maintained entirely with whole cow's milk supplemented with trace minerals and cod liver oil throughout their life span, however, did not develop aortic and gastric lesions (McCay et al., '52). Dreizen et al. ('61) thus concluded that either specific nutrients in butter fat or trace minerals, or both, may act to preserve the integrity of rat smooth muscle.

Considerable research has been conducted by Hill et al. ('44) and other research workers in which an unidentified growth factor for the chick was demonstrated in dried whey. Davis et al. ('62), in studies of an unidentified growth factor in cow's milk which promotes chick growth, showed that dried skim milk was as effective in improving growth as an equivalent level of dried whey.

The studies reported herein were designed to investigate the effect of lactose and dried skim milk upon the magnesium deficiency syndrome of the weanling dog.

EXPERIMENTAL

The composition of the low-magnesium basal diet used in this study (table 1) is similar to that described by Bunce et al. ('62). Magnesium was added to the basal diet as $MgSO_4$. Additions of lactose and $MgSO_4$ were made at the expense of sucrose. The composition of those diets including dried skim milk (28%) was altered to contain equal levels of protein and minerals as the other diets. When 28% of dried skim milk was added, the diet contained 13.6% of lactose and 320 ppm of magnesium. The basal diet contained 50 ppm of magnesium as determined by the method of Mitchell ('54), and also 0.6% of calcium and 0.35% of phosphorus.

Six-week-old Beagle pups from our colony and Shepard-Collie pups, purchased locally, were used in this study and allotted by littermate distribution. Animals were vaccinated against canine distemper, infectious canine hepatitis and leptospiro-

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TABLE 1

Composition of low-magnesium basal ration

	%
Casein, extracted[1]	21.00
Sucrose	65.04
White grease, stabilized[2]	8.70
Salts[3]	5.136
Vitamins[4]	0.124

[1] Extracted with hot ethanol.
[2] Product of Oscar Mayer and Company, Madison, Wisconsin.
[3] For 100 gm of ration: (in grams) KCl, 1.14; NaCl, 1.00; $CaCO_3$, 1.50; Na_2HPO_4, anhydrous, 1.45; and (in milligrams) $Fe_2(SO_4)_3$, 36; $CuSO_4 \cdot 5H_2O$, 2.74; $MnSO_4 \cdot 1H_2O$, 1.54; $ZnCl_2$ (dry), 2.09; $CoCl_2 \cdot 6H_2O$, 0.88; KI, 3.22.
[4] For 100 gm of ration: (in milligrams) thiamine·HCl, 0.132; riboflavin, 0.264; nicotinamide, 0.900; Ca pantothenate, 0.200; pyridoxal·HCl, 0.088; folic acid, 0.030; D-biotin, 0.010; also vitamin B_{12}, 2.2 μg and choline chloride, 0.123 gm; *dl*-α-tocopherol was added to halibut liver oil, and the mixture administered every 3 to 4 days, providing 1 mg to tocopherol, 10 to 15 IU of vitamin A/pound of body weight/day.

sis, and were treated for internal and external parasites when necessary. The dogs were housed in cages with expanded metal floors and fed the diets and distilled water ad libitum. The duration of experiments 1 and 2 was 8 weeks, and that of experiment 3 was 11 weeks.

Blood samples were collected from the jugular vein and allowed to clot. Serum was analyzed for magnesium and calcium by the method of Robinson and Rathbun ('59) and phosphorus by the method of Fiske and Subbarow ('25). Following necropsy, the aorta, kidneys and right femur were removed and frozen. Samples of various tissues were taken for histopathological examination, the results of which are reported by Morris et al. ('63).

After the determination of the dry weight, samples of aorta and kidney were ashed for 18 to 24 hours at 600°C and dissolved in 0.6 N HCl. Calcium and phosphorus determinations were made on aliquots of the solutions by the methods used for serum. Following drying and ether extraction, femurs were ashed at 600°C for 18 hours.

RESULTS AND DISCUSSION

The symptoms of magnesium deficiency and the results of necropsies of dogs fed low magnesium diets are described elsewhere (Morris et al., '63). Symptoms of magnesium deficiency similar to those described by Bunce et al. ('62) were observed in animals in experiments 1 and 2 fed the low magnesium diets. These were anorexia, vasodilatation, muscular weakness, convulsions and ataxia. Convulsions were not observed in animals in experiment 3 that were fed diets containing 100 ppm of magnesium. Muscular weakness was evidenced by a marked bilateral relaxation of the carpus.

Mineralization of various soft tissues was observed at necropsy. The aorta, kidneys, heart, and carotid, coronary and subclavian arteries were the tissues most frequently exhibiting gross mineralization. Mineral deposits were particularly prominent in the first centimeter of the aorta adjacent to the aortic valve. Other tissues in which deposits were noted included the trachea, bronchi, larynx and parietal pleura.

The response of weanling dogs fed a diet containing dried skim milk was compared in experiment 1 with that of dogs fed a diet in which magnesium was supplied as $MgSO_4$ (table 2). The diet supplemented with dried skim milk (28%) contained the same amount of casein (21%) and magnesium (320 ppm) as the diet containing $MgSO_4$. Dogs fed the magnesium-deficient basal diet served as negative controls in this experiment.

Decreased growth and femur ash were observed in the dogs fed the magnesium-deficient basal diet as compared with dogs fed the other two diets. They showed typical deficiency patterns; i.e., decreased concentrations of magnesium and calcium and increased concentrations of phosphorus. The total ash, calcium and phosphorus concentrations of both the aorta and kidney from the magnesium-deficient animals were higher than those of dogs fed the other two diets in this experiment. The increased growth and femur ash of animals fed the diet containing dried skim milk, as compared with dogs fed the diet containing $MgSO_4$ was not statistically different. No differences could be detected in serum, aorta and kidney values of the two groups.

Experiment 2 was designed to study the effect of lactose on the magnesium deficiency syndrome and to determine whether the lactose portion of dried skim milk was responsible for the increased growth observed in experiment 1 as a result of including dried skim milk in the

TABLE 2

Effect of supplementation of $MgSO_4$, lactose and dried skim milk on dogs fed a low magensium diet (50 ppm)

Diet	No. of dogs	Weight gain[1]	Femur ash	Serum analysis		
				Calcium	Phosphorus	Magnesium
			%	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
		Experiment 1				
Basal diet, 50 ppm Mg	5	2.30 ± 0.38[2]	49.7 ± 2.0	7.88 ± 0.90	9.76 ± 0.25	0.89 ± 0.22
Basal + $MgSO_4$, 320 ppm Mg	4	3.10 ± 0.39	50.5 ± 1.5	11.33 ± 0.86	8.47 ± 1.07	1.89 ± 0.08
Basal + dried skim milk, 320 ppm Mg	4	4.07 ± 0.96	51.6 ± 0.6	13.03 ± 0.71	8.48 ± 0.14	1.44 ± 0.31
		Experiment 2				
Basal diet, 50 ppm Mg	8	2.42 ± 0.35	44.7 ± 0.4	7.04 ± 0.40	12.51 ± 0.63	0.51 ± 0.06
Basal + lactose, 13.6%	9	2.85 ± 0.44	44.0 ± 0.8	8.08 ± 0.98	10.74 ± 0.69	0.84 ± 0.16
Basal + $MgSO_4$, 320 ppm + lactose, 13.6%	8	3.43 ± 0.41	47.5 ± 1.2	11.38 ± 0.79	10.55 ± 0.59	1.93 ± 0.11
Basal + dried skim milk, 28%[3]	8	4.19 ± 0.39	51.7 ± 0.4	13.40 ± 0.73	10.19 ± 0.32	1.81 ± 0.07

Diet	Aorta analysis			Kidney analysis		
	Ash	Calcium	Phosphorus	Ash	Calcium	Phosphorus
	mg/100 gm dry aorta	*mg/100 gm dry aorta*	*mg/100 gm dry aorta*	*mg/100 gm dry kidney*	*mg/100 gm dry kidney*	*mg/100 gm dry kidney*
		Experiment 1				
Basal diet, 50 ppm Mg	4070 ± 1028	1076 ± 468	400 ± 50	6152 ± 1005	683 ± 408	488 ± 49
Basal + $MgSO_4$, 320 ppm Mg	2433 ± 260	116 ± 39	256 ± 6	5140 ± 41	174 ± 24	343 ± 29
Basal + dried skim milk, 320 ppm Mg	2160 ± 144	118 ± 13	220 ± 18	5335 ± 149	185 ± 11	385 ± 30
		Experiment 2				
Basal diet, 50 ppm Mg	2841 ± 336	1035 ± 118	424 ± 72	7540 ± 387	1086 ± 162	670 ± 87
Basal + lactose, 13.6%	2152 ± 203	225 ± 45	267 ± 30	6382 ± 458	608 ± 194	360 ± 90
Basal + $MgSO_4$, 320 ppm + lactose, 13.6%	2303 ± 113	166 ± 24	296 ± 28	5450 ± 126	214 ± 17	332 ± 87
Basal + dried skim milk, 28%[3]	2361 ± 115	132 ± 11	275 ± 23	5260 ± 164	224 ± 14	278 ± 83

[1] Weight gain expressed as the number of times the animal increased its original body weight.
[2] Mean ± SE of mean.
[3] Diet contains 320 ppm of magnesium and 13.6% of lactose.

diet. The diets used in this study were: (1) the low magnesium basal diet; (2) the basal diet plus lactose; (3) the basal diet plus lactose and $MgSO_4$; and (4) the basal diet plus dried skim milk. All diets contained the same amounts of casein. The results of this experiment are shown in table 2.

Animals fed the basal diet with added lactose exhibited a slight, but statistically nonsignificant, increase in rate of gain over dogs fed the basal diet alone. The serum calcium and magnesium values of dogs fed the basal diet with added lactose were not as low, nor were the serum phosphorus values as high, as those of dogs fed the low magnesium basal diet. No differences were noted in the femur ash in these two groups. Dogs fed the diet containing lactose and $MgSO_4$ grew more rapidly than either of the two previously mentioned groups and exhibited a higher percentage of femur ash. The serum calcium, phosphorus and magnesium concentrations were all within the normal range. The group fed the diet containing dried skim milk exhibited the highest growth rates and femur ash of any groups in this experiment. The serum concentrations of calcium, phosphorus and magnesium were similar to the values of animals fed the diet containing lactose and $MgSO_4$.

The ash, calcium and phosphorus values of aortas from dogs fed the basal diet were markedly higher than those of animals fed diets adequate in magnesium. The aortas of dogs fed the basal diet plus lactose, however, contained similar amounts of ash, calcium and phosphorus, as did aortas from dogs fed diets adequate in magnesium. The kidneys of dogs fed the basal diet contained increased amounts of ash, calcium and phosphorus when compared with the amounts in kidneys from dogs fed diets adequate in magnesium. The kidneys of dogs fed the basal diet plus lactose were also higher in ash, calcium and phosphorus, but the increase was approximately one-half that of dogs fed the basal diet alone. These results suggested that the inclusion of lactose in the diet resulted in partial prevention of soft tissue calcification. The concentration of phosphorus in the aorta and kidneys of dogs fed diets adequate in magnesium was higher than the concentration of calcium. The reverse was true for the aorta and kidneys of dogs fed the low magnesium basal diet and for the kidneys of dogs fed the basal diet plus lactose. Similar results were also observed in experiment 1. There was considerable variation in the severity of the soft tissue calcification of animals fed the low magnesium diets, particularly in experiment 1, as evidenced by the high standard error of the mean values for aorta and kidney ash, calcium and phosphorus. Dogs fed the diets adequate in magnesium, which had normal aortas and kidneys, exhibited much smaller standard error of the mean values.

Experiment 3 was designed to determine the level at which lactose exerted its effect upon animals fed a submarginal level of magnesium. Weanling pups were fed diets containing 100 ppm of magnesium and zero, 5, 10 and 15% of lactose. The growth and serum data are presented in figure 1. The groups fed diets containing 10 and 15% of lactose gained more rapidly and consistently than did those dogs fed diets containing zero and 5% of lactose. As was pointed out previously, the serum calcium and magnesium values decrease and the serum phosphorus values increase in magnesium deficient dogs as compared with values of dogs fed diets adequate in magnesium. The mineral concentration of serum of dogs fed diets containing 10 and 15% of lactose in experiment 3 suggested a more severe magnesium deficiency than did the serum mineral concentrations of dogs fed diets containing zero and 5% of lactose; that is, they exhibited lower serum magnesium and calcium values and higher serum phosphorus values. These results suggested that larger amounts of magnesium were being removed from the blood by the more rapidly growing dogs, thus making the deficiency somewhat more acute, or, as suggested by Forbes ('61), the excretion of this mineral was enhanced by the higher levels of dietary lactose.

Femur, aorta and kidney analyses failed to reveal any distinct differences resulting from the addition of various levels of lactose to the diet. The average values for growth, serum and tissue data of all dogs in experiment 3 are shown in table

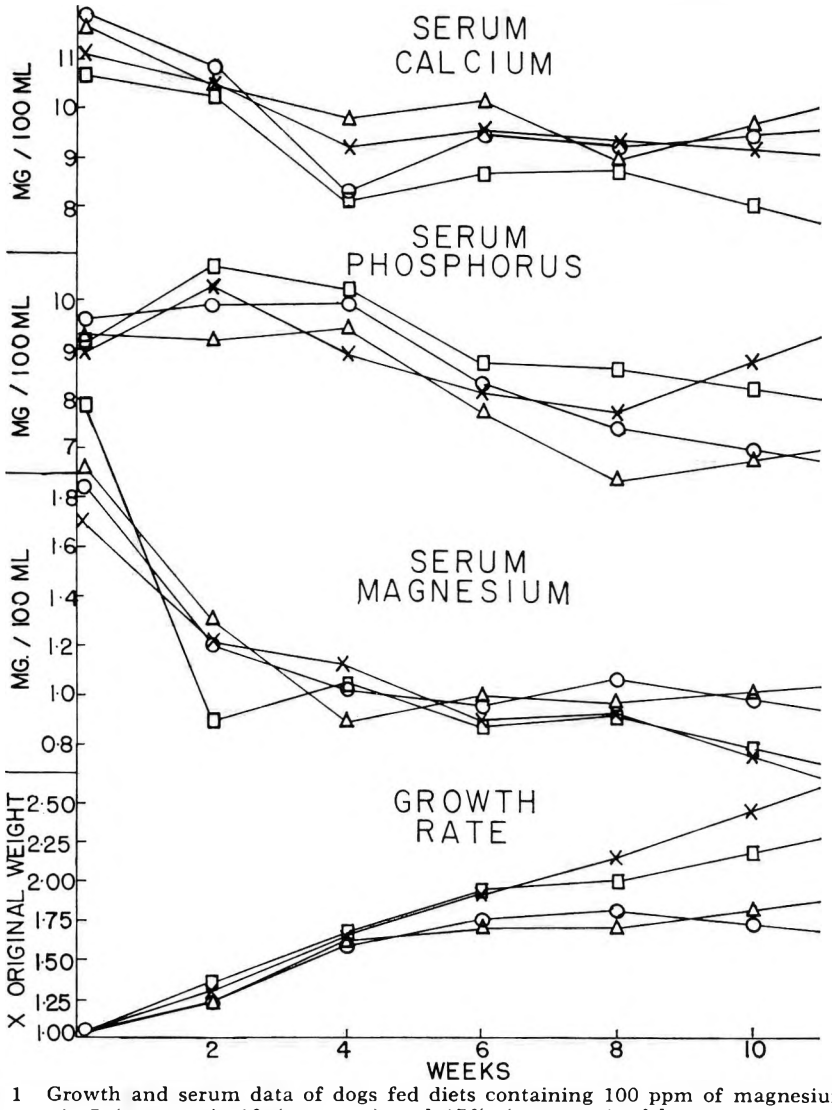

Fig. 1 Growth and serum data of dogs fed diets containing 100 ppm of magnesium and 0 (○——○), 5 (△——△), 10 (×——×) and 15% (□——□) of lactose.

3. Serum and kidney analyses revealed a less severe deficient state than noted in experiments 1 and 2 which appears to be consistent with the inclusion of the additional 50 ppm of magnesium in the diet. Results of aorta analyses were similar to those observed for dogs fed the low magnesium basal diet. However, the experimental period in experiment 3 was 11 weeks as compared with 8 weeks for experiments 1 and 2.

Although the aortas and kidneys of dogs fed diets containing $MgSO_4$ and lactose contained similar amounts of ash, calcium and phosphorus as those exhibited by aortas and kidneys of dogs fed diets containing dried skim milk, growth rate and femur ash were not as high. Since these two diets contained the same amounts of protein, lactose and minerals, the increased growth and femur ash of dogs fed the diet containing dried skim milk indicated that either the minerals in dried skim milk were more utilizable than the inorganic salts in the diet containing $MgSO_4$ plus lactose, or that dried skim

TABLE 3

Average values for growth, serum and tissue data of all dogs in experiment 3 that were fed diets containing 100 ppm of magnesium[1]

Weight gain[2]	2.16 ± 0.16[3]
Serum calcium, mg/100 ml	8.78 ± 0.57
Serum phosphorus, mg/100 ml	7.40 ± 0.33
Serum magnesium, mg/100 ml	0.88 ± 0.07
Femur ash, %	55.5 ± 0.60
Aorta ash, mg/100 gm dry aorta	3996 ± 459
Aorta calcium, mg/100 gm dry aorta	802 ± 167
Aorta phosphorus, mg/100 gm dry aorta	598 ± 84
Kidney ash, mg/100 gm dry kidney	5345 ± 127
Kidney calcium, mg/100 gm dry kidney	234 ± 26
Kidney phosphorus, mg/100 gm dry kidney	542 ± 37

[1] Averages of values from 15 dogs. Serum values are shown for dogs after 8 weeks on experiment.
[2] Weight gain expressed as the number of times the animal increased its original body weight.
[3] Mean ± SE of mean.

milk contained an unidentified growth factor.

Growth rate and femur ash appeared to be more sensitive indicators of the adequacy of the diet than were aorta and kidney analyses, which distingiushed only severely magnesuim deficient tissue from normal tissue.

SUMMARY

Studies were conducted to investigate the effect of lactose and dried skim milk upon the magnesium deficiency syndrome of the weanling dog. Dogs fed low magnesium diets exhibited decreased growth, femur ash, serum calcium and magnesium, and increased serum phosphorus values as compared with those of dogs fed diets adequate in magnesium. Aorta and kidney ash, and the calcium and phosphorus values of these dogs were increased. Both dried skim milk and $MgSO_4$ were shown to furnish adequate amounts of magnesium to support growth and prevent soft tissue calcification. Dogs fed diets containing dried skim milk exhibited more rapid growth and higher femur ash than dogs fed diets containing similar amounts of lactose and magnesium from $MgSO_4$. Ash, calcium and phosphorus values of aortas and kidneys of dogs fed the low magnesium basal diet with added lactose indicated less severe calcification than that exhibited by dogs fed the low magnesium basal diet alone. Dogs fed diets marginally adequate in magnesium (100 ppm) grew more rapidly when the diet contained 10% of lactose than when higher or lower amounts were included in the diet.

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Influence of Lactose and Dried Skim Milk upon the Magnesium Deficiency Syndrome in the Dog

II. PATHOLOGICAL CHANGES[1]

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Morphological changes produced by low magnesium diets have been described by Greenberg et al. ('36), Lowenhaupt et al. ('50), MacIntyre et al. ('58) and Ko et al. ('62) in rats; Barron et al. ('49) in rats and rabibts; Bird et al. ('49) in chicks; and Blaxter et al. ('54) and Moore et al. ('38) in calves.

Following the description of the symptomatology by Orent et al. ('32), little was published concerning the magnesium deficiency syndrome in dogs until the work of Syllm-Rapoport et al. ('58) and Unglaub et al. ('59) describing clinical symptoms, chemical changes in serum and pathological findings in 16 dogs consuming a diet containing 5 ppm of magnesium. These animals exhibited calcification of the larynx, trachea, lungs, kidneys and cardiovascular system.

The studies reported herein were designed to observe the effect of lactose and dried skim milk upon the magnesium deficiency syndrome and to further investigate the pathological changes produced in dogs by low magnesium diets.

EXPERIMENTAL

The diet, husbandry, methods and values obtained on chemical examination of the tissues were the same as those reported by Featherston et al. ('63). Sixty-one dogs were used in three experiments. In experiments 1 and 2 the low magnesium diets contained 50 ppm of magnesium, whereas in experiment 3 all diets contained 100 ppm in an attempt to prevent the symptoms and pathological changes produced by low magnesium diets.

All animals were destroyed, and necropsies were performed immediately. Tissues were placed in the refrigerator in buffered formaldehyde USP diluted 1:10; embedded in paraffin; sectioned at 7 μ; and stained with Harris's hematoxylin and eosin Y, a combination of alcian blue and periodic acid Schiff (PAS), toluidine blue and by the von Kossa method for calcium.

RESULTS AND DISCUSSION

Symptoms of magnesium deficiency in the dog similar to those described by Orent et al. ('32) and Syllm-Rapoport et al. ('58) were observed in animals consuming the low magnesium diets. Vasodilatation of the skin and tongue appeared during the second week in deficient animals in experiments 1 and 2, and during the fourth week in experiment 3. It occurred later over the entire body, but was particularly evident on the ventral surface of the abdomen, on the base of nonpigmented toenails and on the tongue. Alopecia was observed around the eyes and mouth of deficient dogs. Excoriations of the skin were not noted. The absence of this sign may be due to the higher levels of magnesium in the diets in this study than in those used by other workers. Muscular weakness was evidenced by a marked bilateral relaxation of the carpus and tarsus. The severity of this condition increased as the deficiency and the degree of relaxation at any time was correlated with the magnesium content of the diet;

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i.e., the animals in experiment 3 consuming diets containing 100 ppm of magnesium exhibited only spreading of the toes and slight muscular relaxation, whereas those in experiments 1 and 2 walked on the posterior surface of the metacarpus.

During the second week the test animals in experiments 1 and 2 became apathetic and were content to lie quietly in the cage. Nervousness and convulsions were observed as early as the third week in experiments 1 and 2. Nervousness was observed in experiment 3, but convulsions were not seen in any of the animals fed diets containing 100 ppm of magnesium.

Gross pathology. At necropsy gross pathological changes, consisting of mineral deposits, were observed in the heart, kidneys, trachea, larynx, bronchi, aorta, carotid, coronary, and subclavian arteries, and under the parietal pleura between the ribs. These changes have been described by Syllm-Rapoport et al. ('58). Hard, raised, white plaques were particularly prominent under the endothelium in the first centimeter of the aorta, lateral to the cusps of the aortic valve. These plaques were present in all animals consuming deficient diets.

As noted by chemical analysis and gross observation (table 1), the addition of lactose to the basal diet in experiment 2 and 50 ppm magnesium in experiment 3 slightly reduced the incidence of soft tissue calcification. The severity of the changes was greatly diminished in experiment 3 by the addition of magnesium in the diet. Seven of these animals had grossly normal aortas except for the first centimeter which was calcified in all 15 animals. Calcification of the kidney was seen in only 2 of the 15 animals. No animals consuming diets supplemented with either $MgSO_4$ or dried skim milk exhibited gross pathological changes.

Histopathology. All animals fed the magnesium-deficient diets exhibited significant histopathological changes. Calcification of blood vessels was the primary change. It was observed in all arteries including the large elastic arteries, medium sized arteries, small arteries, arterioles and capillaries in the renal medulla, lung and uterus. Lesions were observed in vessels supplying the thyroid, stomach, adrenal, pancreas, salivary gland, uterus, bladder and intestine.

In the kidney medial calcification was particularly severe in the interlobular arteries in the cortex, but was observed throughout the arterial tree. The lesions ranged from small mineral deposits along the internal elastic membrane to calcification of the entire vessel wall. Some of the vessels were completely occluded, but often a severely calcified vessel showed no reduction in the size of its lumen. These interlobular arteries were often surrounded by an inflammatory reaction consisting of macrophages, lymphocytes and neutrophils (fig. 1). The reaction varied in intensity depending upon the extent to which the vessel was calcified. Many of the kidneys from animals in experiment 3

TABLE 1

Incidence of gross calcification

Diet	Heart	Aorta	Aortic valve	Trachea	Carotid arteries	Kidneys
			Experiment 1			
Basal diet (50 ppm Mg)	3/4[1]	3/4	4/4	1/4	3/4	2/4
			Experiment 2			
Basal diet (50 ppm Mg)	3/4	4/4	4/4	1/4	4/4	4/4
Basal diet + lactose (50 ppm Mg)	2/4	4/4	4/4	1/4	1/4	0/4
			Experiment 3			
Basal diet + $MgSO_4$ (100 ppm Mg)	11/15	8/15	15/15	1/15	7/15	2/15

[1] Ratio of animals affected to total examined.

showed only slight calcification of the kidney arteries and no inflammatory reaction. In the medulla of the kidney, small calcific casts were present in the arteriolae rectae (fig. 2). This lesion was noted only in animals in experiments 1 and 2 and not in the less deficient dogs in experiment 3. No lesions were seen in the tubules.

In all experiments, calcification of the aorta and large elastic arteries was similar to that described by Moore et al. ('38) in calves. It appeared to be randomly distributed throughout the intima and media. There was no preference for the media adjacent to the adventitia as reported by Unglaub et al. ('59). In moderately deficient animals most lesions in the large elastic arteries involved the internal elastic membrane, but in severe cases mineral deposits were observed at all levels of the media. In all cases the mineral appeared to be precipitated on the external surface of the elastic fibers. The endothelial cells of the vasa vasorum in the aorta were hypertrophied.

The aorta and other large elastic arteries of many of the animals in experiment 3, consuming diets containing 100 ppm of magnesium, exhibited focal areas of mucoid degeneration with increased amounts of ground substance. These processes resulted in separation of the elastic fibers and were found at all levels of the intima and media (fig. 3).

Some of the above foci were immediately adjacent to and associated with small mineral deposits, whereas others showed no approximation to any mineral. A pronounced metachromasia with toluidine blue and a bright turquoise-blue with alcian blue, not seen in other areas of the same aorta or in control animals, suggested the presence of increased amounts of acid mucopolysaccharides. Some of these areas contained von Kossa positive material which appeared as fine black stippling throughout the focus, indicating the presence of calcium salts (fig. 4), while others did not contain von Kossa positive material.

The alcian blue-PAS stains of the aorta revealed PAS-positive material in and around the large mineral deposits. This increase in the PAS stainability has been observed in other tissues undergoing calcification. The degenerative areas did not exhibit a positive PAS reaction.

In medum-size muscle and organ arteries, calcification was observed on the internal elastic membrane in the less deficient animals in experiment 3, but in severely deficient dogs in experiment 2 the lesions had expanded to involve the entire media. No calcification of the adventitia was noted in these vessels.

In the heart extensive calcification was seen involving the subendocardial elastic fibers and the muscle fibers of the myocardium. Such mineral deposits were frequently associated with blood vessels and were often surrounded by fibroblasts or infiltrated with young connective tissue.

Calcification was frequently seen involving one or more muscle fibers immediately adjacent to a small coronary arteriole which was undergoing degeneration (fig. 5). These arterioles were surrounded by increased amounts of metachromatic material. Degenerative changes were not seen in the large coronary arteries. Several severely deficient dogs had large myocardial infarcts.

Calcification was observed in the larynx, trachea, bronchi and lungs. In the layrnx, trachea and bronchi the mineral was deposited along the elastic fibers in the submucosa. Frequently it expanded to involve the mucosa which became denuded. In severe cases the deposits were surrounded by fibroblasts and young connective tissue. In the lung, rod shaped calcific casts were observed in the capillaries in the interalveolar spaces. Many of these casts had expanded beyond the vessel wall into the surrounding stroma. When sectioned longitudinally these casts appeared to follow the course of the vessel.

Sections of the diaphragm and other voluntary muscles revealed degenerative changes characterized by swelling and fragmentation of muscle fibers, loss of striation, proliferation of sarcolemmal sheath cells and infiltration of inflammatory cells. Small numbers of inflammatory cells surrounded the blood vessels in the areas of degenerating muscle (fig. 6). It was not determined whether these cells were due to a primary arteritis or were secondary to the degenerating muscle.

Calcification of smooth muscle was noted in the tunica muscularis of the stomach of two animals. The deposits were closely related to degenerating arterioles, which were surrounded by an inflammatory infiltrate composed of mononuclear cells.

In one animal calcific casts were noted in the capillaries immediately under the endometrium, and scattered throughout the internal one-third of the myometrium of the uterus. Some of these casts had expanded and involved the surrounding muscle fibers.

Calcification of the spleen was observed in two dogs. In each instance the lesion involved the wall of the central artery in the splenic corpuscle. No calcific deposits were seen in the capsule or trabeculae of the spleen.

Decalcified sections of the radius and ulna of dogs with severe magnesium deficiency revealed lines of interrupted mineralization, sealing off of the epiphyseal cartilage and failure to form a calcification lattice. Such changes are nonspecific and have been described by Follis ('58) to be a result of inanition among others. Due to their reduced food intake and irregular rate of growth, these changes were to be expected in the deficient dogs.

Examination of liver, thyroid, parathyroid, small and large intestine, bladder, gonads, adrenals, lymph nodes, pancreas, thymus, salivary glands, pituitary, brain and sections taken at various levels of the suspensary apparatus of the limbs revealed no difference from control animals. No histopathological changes were observed in any dogs in experiments 1 and 2 consuming the basal diet supplemented with dried skim milk or 320 ppm of $MgSO_4$. In experiment 2 no difference was observed microscopically between animals consuming the basal diet and those consuming the basal diet supplemented with lactose.

The extreme variation in the pathological changes which have been reported by other workers in various species may be due in part to variation in dietary ingredients and levels of these ingredients in the diets used to produce changes, as well as to the inherent variation between species.

Several of the pathological changes observed in this study, using diets containing 50 and 100 ppm of magnesium, have not been reported earlier with diets containing 5 ppm of magnesium or less. The importance of the relationship between the level of magnesium in the diet to the morphological changes produced has been emphasized in this study where the more severely deficient dogs in experiments 1 and 2 exhibited extensive calcification of soft tissue, whereas the less deficient animals in experiment 3 exhibited changes preceding the calcification process not observed in the earlier experiments. Follis ('58) has stated, "When an acute, overwhelming deficiency state is produced, the morphologic changes may be slight or even absent in comparison with tissue alterations which may be encountered when the deficiency is more chronic and therefore not so severe."

The finding of foci of increased ground substance in the large elastic arteries, exhibiting metachromasia and a positive alcian blue reaction, suggests that magnesium may exert an effect on the integrity of mucopolysaccharides in the body. The degeneration and calcification of arterioles and capillaries associated with calcification of the heart and smooth muscle suggests that vascular changes may play an important role in the development of lesions in these organs. The fact that calcification of the capillaries in the lung, uterus and renal medulla had little effect on surrounding structures may be due to the extensive collateral circulation in these organs.

These findings give reason to believe that the calcification of soft tissue observed in chronic magnesium deficiency in the dog is not purely metastatic as has been postulated, but rather a form of dystrophic calcification where the calcification process is preceded by degenerative changes.

SUMMARY

In the dog low magnesium diets cause widespread calcification of the kidney, heart, blood vessels, larynx, trachea, lungs and smooth muscle of the stomach and uterus and degeneration of skeletal muscles. Dogs consuming diets marginally deficient in magnesium exhibited degenera-

tive changes in the blood vessels. The addition of dried skim milk or $MgSO_4$ to the diet completely prevented the development of magnesium deficiency. Special stains indicated that magnesium may exert an effect on the integrity of mucopolysaccharides in the body. The relationship between morphological changes produced and the level of dietary magnesium was discussed. Results indicated that the calcification observed in magnesium deficiency in the dog may be a form of dystrophic calcification.

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PLATE 1

EXPLANATION OF FIGURES

1 Kidney from a magnesium-deficient dog. Severely calcified interlobular artery surrounded by mononuclear cells. H & E. × 125.

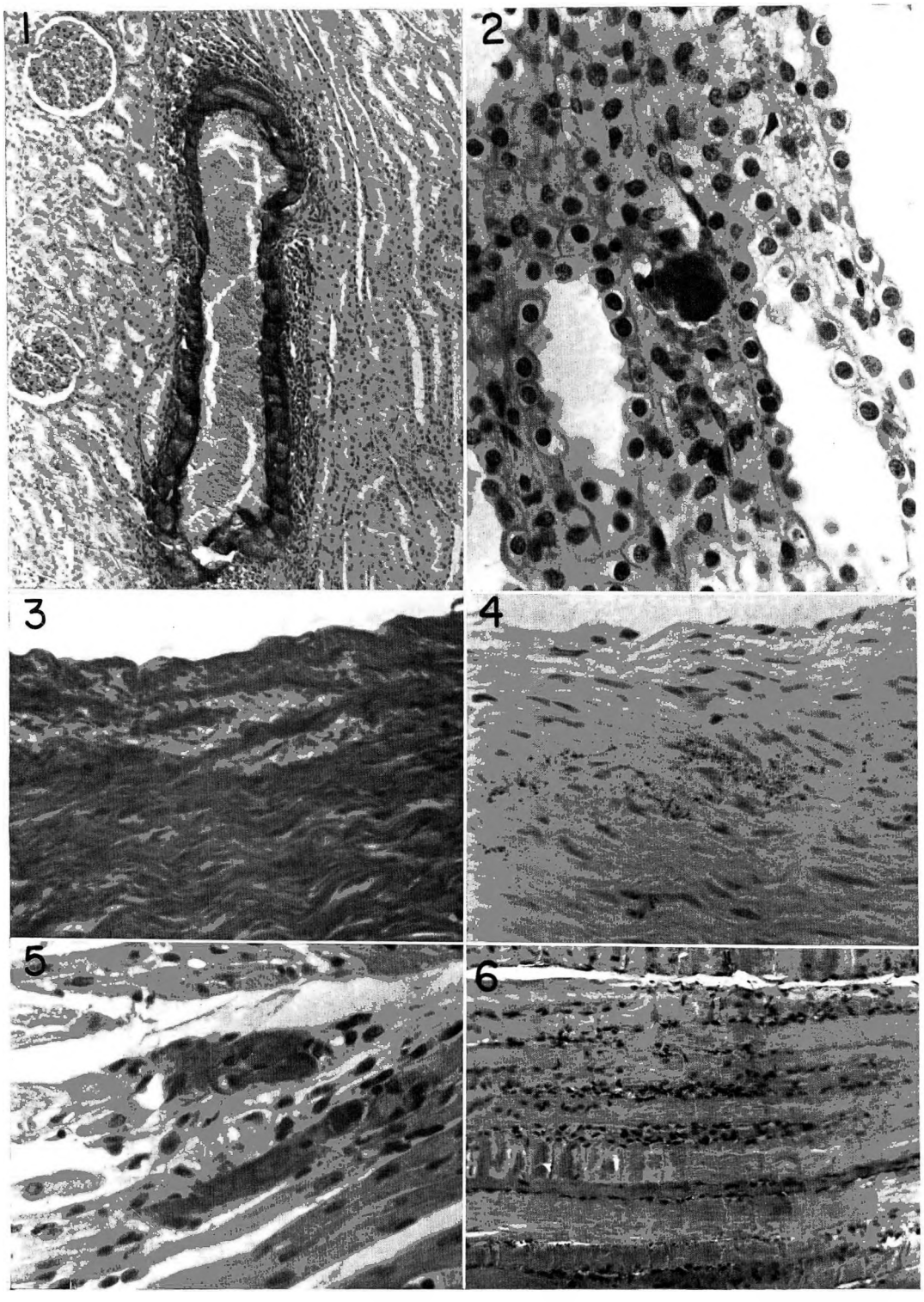
2 Kidney from a magnesium-deficient dog. Calcific cast within the lumen of a capillary in the renal medulla. H & E. × 500.

3 Aorta from a magnesium-deficient dog. Foci of mucoid degeneration producing separation of the elastic fibers. Alcian blue-PAS. × 500.

4 Aorta from a magnesium-deficient dog. Tiny deposits of mineral present in the foci of mucoid degeneration. von Kossa. × 500.

5 Heart from a magnesium-deficient dog. Two calcified muscle fibers surrounding remnants of a degenerating coronary arteriole. H & E. × 500.

6 Skeletal muscle from a magnesium-deficient dog. Degenerating muscle fibers separated by an infiltration of mononuclear cells. H & E. × 125.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Production of Hypercholesterolemia and Atherosclerosis by a Diet Rich in Shellfish[1]

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Current emphasis in the treatment and prevention of atherosclerosis has led to the use of diets low in saturated fat and high in polyunsaturated fat content (Brown and Page, '58; Jolliffe et al., '59; Stamler, '60). Viewed in this context, most sea foods have an ideal fatty acid composition. They are low in saturated fat, and unsaturated fatty acids constitute up to 80% or more of the total fat content. Most of the unsaturated fatty acids found in marine animals are in the polyunsaturated category (Hilditch, '56). Ahrens et al. ('59), showed that human subjects consuming a fish oil, menhaden, had definite lowering of their serum cholesterol levels. Such an experimental result has confirmed the apparent desirability of the fatty acid composition of sea foods. Because of these considerations, sea foods, including shellfish, are commonly recommended in diets designed to reduce the serum cholesterol concentration and to treat atherosclerosis in man.[3]

Although many different foods and fats have been fed to humans and animals to determine their effects upon lipid metabolism and atherosclerosis, the consequences of feeding the meat of sea food rather than specific fish oils are not well documented. In the study to be reported we have asked two questions: first, will a diet high in shellfish influence the serum cholesterol level, and second, will the long-term consumption of such a diet ultimately produce atherosclerosis in an experimental animal?

MATERIALS AND METHODS

Mature, male New Zealand white rabbits which had been maintained with commercial rabbit chow[4] were divided into three groups. Eleven rabbits were fed a special chow containing a mixture of dried shrimp meal[5] and the commercial chow for 24 weeks. The special chow was compounded into pellets[4] with equal parts of shrimp meal and the commercial chow. The shrimp meal had been prepared from freeze-dried fresh shrimp. It contained about 2% of water as compared with the usual 67% of water content of fresh shrimp (Turner, '59). The rabbits ate these pellets readily. In a trial run, to determine the feasibiliy of this kind of feeding, rabbits had previously rejected shrimp provided in small pieces or finely ground.

Two other groups of rabbits were used as control groups and were given other feeding regimens. Five rabbits were fed the commercial rabbit chow only. This chow is prepared entirely from vegetable sources and contains no cholesterol. Six rabbits were fed the commercial chow plus 0.25% crystalline cholesterol and 18.5% corn oil.

Blood was drawn periodically for lipid analyses by needle puncture of the central artery of the ear. The serum cholesterol was determined in duplicate by the method of Abell et al. ('52) as modified by Anderson and Keys ('56), and the serum triglyceride by the method of Van Handel and Zilversmit ('57).

After 24 weeks of feeding, the animals were killed with sodium pentobarbital and the aortae and hearts removed en bloc.

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[2] Research fellow, American and Iowa Heart Associations.

[3] The authors have surveyed 18 recently published articles and books listing diets for the treatment of coronary heart disease. Without exception, all suggest the preferential use of fish and shellfish (references supplied on request).

[4] Purina Rabbit Chow, The Ralston Purina Company, St. Louis, Missouri.

[5] Supplied by United Fruit and Food Corporation, Boston.

Aortic atheromas were graded from zero to 4 + according to the average extent of intimal involvement. The aortic valve, the ascending thoracic aorta, the descending thoracic aorta, and the abdominal aorta were graded separately. To obtain a more exact expression of atheroma formation, the cholesterol content of these aortae was analyzed. A uniform length of aorta from the aortic valve to diaphram was excised and cleaned of extraneous tissue. The specimen was then dried at 105°C until it reached a constant weight. It was then ground to a fine powder and extracted in 20 parts of a 2:1 chloroform-methanol overnight. Final extraction was accomplished by bringing the mixture to a boil. The residual material was then extracted two more times to make certain that all of the cholesterol had been obtained. Less than 1% of the total cholesterol was obtained by the third extraction. The chloroform-methanol extracts of the aortae were evaporated to dryness and the cholesterol content then measured as above. The cholesterol content of the freeze-dried shrimp was ascertained after a similar extraction procedure.

RESULTS

Shrimp feeding produced a prompt elevation in the serum cholesterol concentrations which was sustained for the duration of the experiment (figs. 1 and 2). After 3.5 weeks of feeding, the mean serum cholesterol had increased from 57 to 438 mg/100 ml. At the end of the experiment, after 24 weeks of feeding, the mean serum cholesterol was 795 mg/100 ml. All rabbits showed a hypercholesterolemic response, but there was great variation in its magnitude. As illustrated in figures 1 and 2, rabbits could be divided into high and low responders. The serum cholesterol concentrations of most rabbits became greatly elevated after a few weeks of shrimp feeding. Note the relative resistance manifested by rabbits 5 and 10. The serum cholesterol level of rabbit 5, for example, did not increase over the baseline until 10.5 weeks of feeding and then fluctuated around an average level of 140 mg/100 ml for the duration of the experiment. Rabbit 10 was slightly less resistant with a final serum cholesterol determination of 235 mg/100 ml. The average weight gain per rabbit was 0.25 kg for

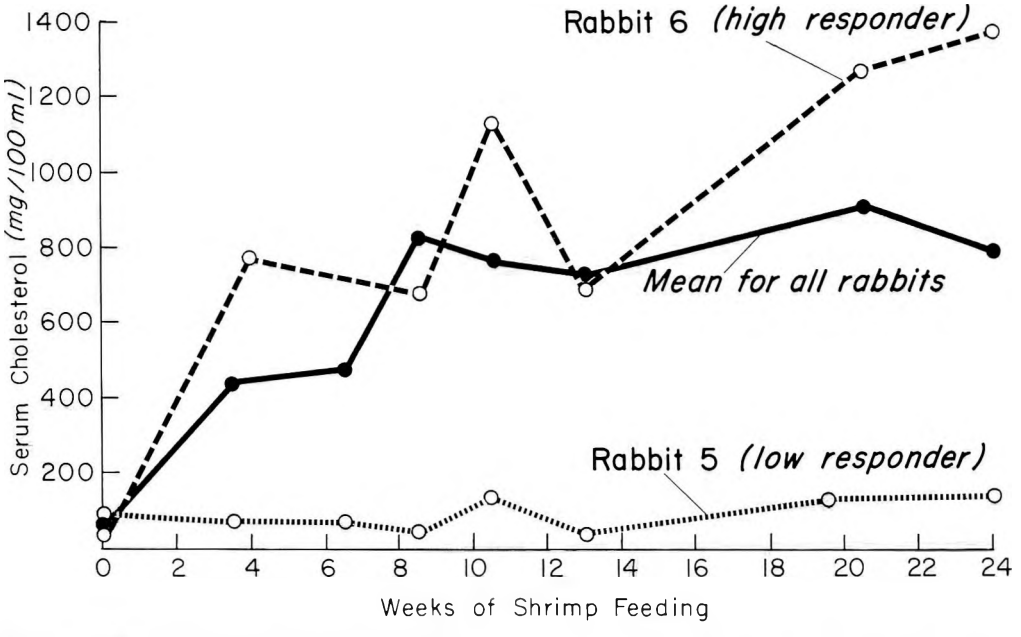

Fig. 1 Serum cholesterol values obtained at intervals during the period of shrimp feeding. Mean values for all rabbits as well as examples of rabbits showing widely differing responses are shown. The rabbits with the highest and lowest aortic cholesterol content (table 1) were selected as examples of high and low responders.

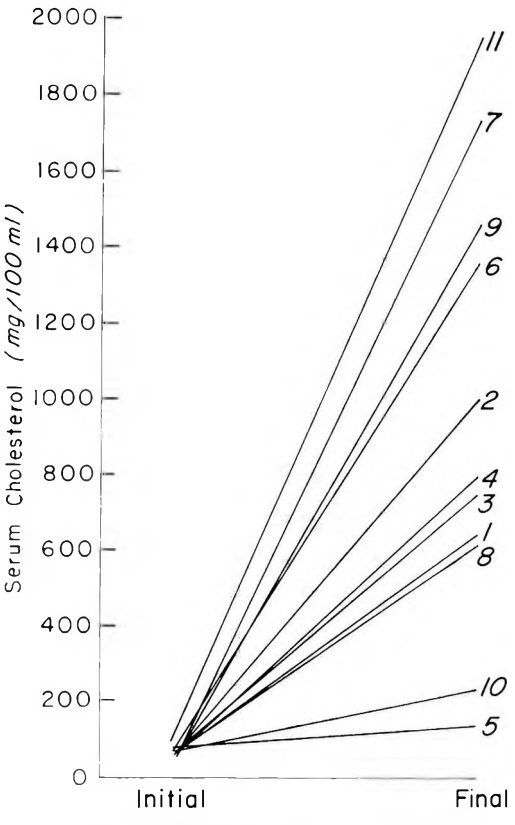

Fig. 2 The initial and final serum cholesterol concentrations for each rabbit consuming the shrimp-commercial chow mixture.

the experimental period and was similar for high and low responders.

The serum from rabbits fed only the commercial chow had a clear appearance. After 3.5 weeks of shrimp feeding the serum from most of the rabbits was milky or lipemic. The cholesterol-fed rabbits also had lactescent serum. The serum triglyceride levels of the shrimp-fed rabbits increased threefold during the study. The mean initial value was 83 mg/100 ml and the mean final value was 266 mg/100 ml. The sole exception to the general tendency of the serum triglyceride to increase was the resistant rabbit 5 whose final value was no greater than the initial one.

At autopsy all shrimp-fed rabbits had gross evidence of atherosclerosis (table 1). The lesions varied from a few fatty streaks to florid, confluent lesions involving most of the initimal surface area. The specimen of aorta shown in figure 3 is typical of this severe involvement. The aortic valve and arch had the greatest lesions; in this instance their entire intimal surfaces had deposits. The lower thoracic and abdominal aorta always had a lesser degree of atherosclerosis than the proximal segments.

The extent of the atheromatous deposits correlated well with the cholesterol

TABLE 1

Effects of shrimp feeding upon the serum cholesterol, aortic atherosclerosis, and aortic cholesterol content

Rabbit	Serum cholesterol		Aortic atherosclerosis (average grade)	Aortic cholesterol content
	Initial	Final		
	mg/100 ml	*mg/100 ml*		*mg/gm dried tissue*
1	66	642	3.3	92.7
2[1]	58	1010	trace[1]	
3	46	754	2.0	60.2
4	46	810	1.7	25.4
5	99	141	trace	5.0
6	57	1393	3.0	135.5
7	49	1745	2.7	130.4
8	26	646	1.3	39.0
9	82	1480	3.0	
10	37	235	trace	7.7
11	65	1930	2.0	72.5
Control[2]	68 ± 21	43 ± 14	0	4.5 ± 0.3
Control[3]	65 ± 12	429 ± 268	1.2	17.0 ± 13.4

[1] Killed after 8 weeks because of accidental femoral fracture.
[2] Rabbits fed only commercial rabbit chow. Mean values and SD.
[3] Rabbits fed commercial chow plus cholesterol in corn oil.

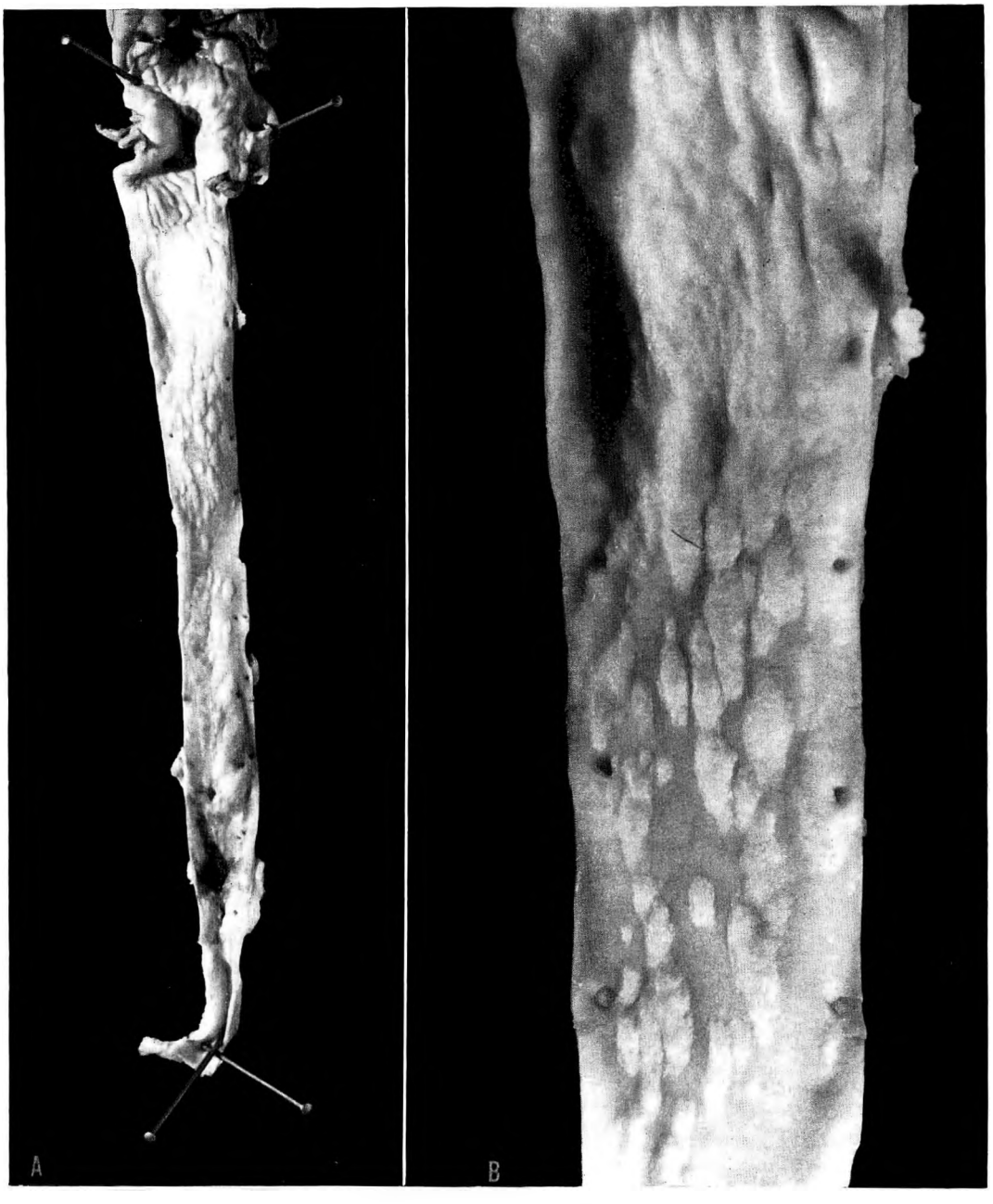

Fig. 3 A typical example of aortic atherosclerosis (rabbit 9) which occurred after 24 weeks of shrimp-commercial chow feeding. Figure 3-A shows the entire specimen with the aortic valve superiorly. Figure 3-B is a close-up of a segment of thoracic aorta. The upper portion of the vessel is most severely involved and the lower portion of the region of the intercostal arteries has patchy lesions between small areas of normal intima.

content of the rabbit aortae (table 1). All shrimp-fed rabbits had an increased amount of aortic cholesterol compared with the control aortae of rabbits fed the commercial chow. For example, rabbit 6 had 135.5 mg of cholesterol/gm of aorta versus the control figure of 4.5. Those rabbits with the highest final serum cholesterol concentrations tended to have the greatest aortic cholesterol accumulation (fig. 4) as well as the most atherosclerotic lesions by gross inspection. Rabbits 5 and 10, the low responders, had only a trace of gross atherosclerosis and also the lowest values for aortic cholesterol, 5.0 and 7.7 mg/gm of aorta. Rabbit 2 had marked hypercholesterolemia but only a few gross lesions. This animal was killed after only 8 weeks of shrimp feeding because it fractured a hind limb after a fall.

The control rabbits fed only vegetable foods in the form of commercial chow did not develop either hypercholesterolemia or atherosclerosis (table 1). As many workers have reported previously (review, Katz and Stamler, '53), the addition of cholesterol dissolved in oil to the diet of rabbits invariably produces hypercholesterolemia and atherosclerosis. All of our cholesterol-fed control rabbits had hypercholesterolemia and atherosclerosis (table 1).

The cholesterol content of different samples of the freeze-dried shrimp used in this study varied from 901 to 910 mg/100 gm of dry weight. Other shrimp from a different source (canned, water packed) contained 814 to 838 mg of cholesterol/100 gm of dry weight. Analyses of the cholesterol content of shrimp in the literature vary greatly. Converted to dry weight on the basis of 67% of water content, these analyses range from 414 mg/100 gm (Kritchevsky and Tepper, '61) to 777 mg/100 gm (Kaucher et al., '43). Phil ('52) obtained the equivalent of 678 mg/100 gm. The reasons for these wide differences are not readily apparent. They may represent differences in shrimp obtained from different waters and at different times of the year. They may, however, be dependent upon completeness of the extraction procedure or upon variations in water content of the shrimp analyzed. We obtained only 801 mg/100 gm

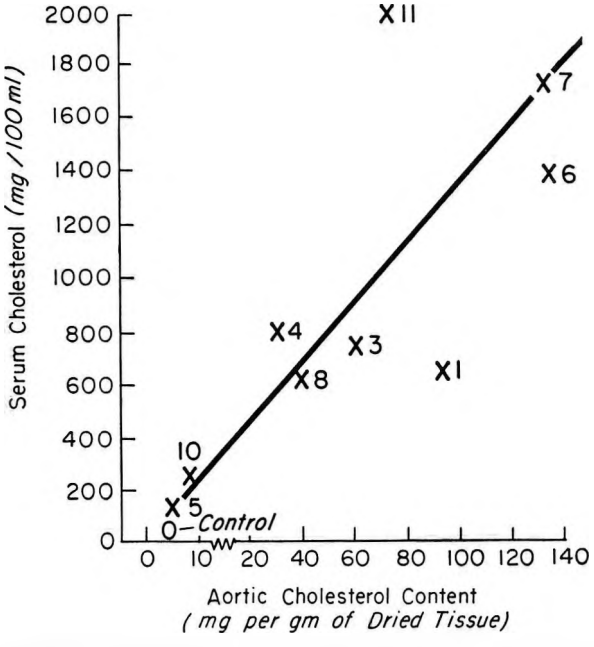

Fig. 4 The correlation between the final serum cholesterol value and the aortic cholesterol content for each rabbit ($r = 0.752$, $P < 0.05$). Each experimental rabbit is represented by its appropriate numerical designation placed adjacent to the symbol X; control rabbits, fed only the commercial chow, are designated by the open circle.

after a prolonged first extraction, a value similar to the 777 mg/100 gm obtained by Kaucher ('43). Since the cholesterol present in animal tissue is intimately associated with the cell wall and other cellular constituents, possibly it is not readily removed by extraction procedures. Furthermore, shrimp has a variable water content. We found that the freeze-dried shrimp placed in water would increase in weight 5 times, thus suggesting that the water content of shrimp might be as high as 80% (Okey, '45). If so, then our analysis of cholesterol content for wet weight would be about 180 mg/100 gm.

DISCUSSION

The constituent of shrimp that produced hypercholesterolemia and extensive atherosclerosis in these animals was probably its cholesterol content. In fact, cholesterol (or related sterols) is almost an obligatory dietary substance in regimens designed to produce atherosclerosis in the experimental animal (Katz and Stamler, '53; Katz et al., '58). The characteristic sterol of crustaceans (shrimp, crabs, lobsters) is cholesterol (Bergmann, '49) and over 90% of the total cholesterol of shrimp is free cholesterol (Kaucher et al., '43; Kritchevisky and Tepper, '61). The amount of cholesterol in the shrimp fed to these rabbits, considering the range of all analyses, both obtained by us and reviewed from the literature, was between 0.21 and 0.45 gm/100 gm of chow. This amount of cholesterol commonly produces atherosclerosis when fed to rabbits. As Portman and Stare ('59) and Mann ('61) have pointed out, the lower range of these values does not greatly differ in terms of total calories consumed, from the amounts of cholesterol contained in some human diets.

Dried shrimp also contains 2.24% neutral fat and 3.89% phospholipid (Kaucher, '43). Shrimp oil has only 13% saturated fat, the remainder probably being largely polyunsaturated (Hilditch, '56) as is common for other sea foods. Thus the polyunsaturated fat present in shrimp did not prevent the extensive atherosclerosis that occurred with its feeding.

In the dried state, shrimp contains about 67% protein (Turner, '59). The rabbits in this study consequently were fed a high protein diet. Diets low in protein or deficient in certain amino acids contribute to the development of atherosclerosis in animals (Mann, '61). On the other hand, large amounts of dietary protein at times reduce the amount of experimental atherosclerosis which customarily occurs with a given diet (Katz et al., '58). From the evidence, therefore, the hypercholesterolemia and atherosclerosis occurring in the shrimp-fed rabbits would not be attributable to the high protein content of the diet.

The implications of this study with respect to diets designed to prevent and treat atherosclerosis in man are obviously indirect. It should be emphasized, however, that the basic problem in atherosclerosis is a diseased artery. In particular, recent investigations have stressed the similarity between experimental atherosclerosis and the disease in man. Myocardial infarction has been produced in the monkey (Cox et al., '58; Taylor et al., '62) and in the rabbit (Myasnikov et al., '58; Myasnikov et al., '61) after the feeding of diets rich in cholesterol and fat. The arterial lesions in these animals have a very close resemblence to the lesions in advanced atherosclerotic disease in man (Constantinides et al., '61; Taylor et al., '62).

Treatment directed at a lowering of the serum lipids does not necessarily lessen the atherosclerotic lesion. In fact, a lowering of the serum cholesterol in the rat from corn oil in the diet has been accompanied by an increased cholesterol content in the aorta as well as in the liver, heart, muscle and intestine (Gerson et al., '61). Because this arterial disease in humans cannot be examined readily to note any effects of dietary alterations, it is necessary, as in other nutritional problems, to use experimental animals to gain information about possible effects of a given food in man.

If the feeding of a certain foodstuff produces extensive atherosclerosis in an animal, then it might follow that this type of food might well be restricted in human diets designed to prevent atherosclerosis in certain susceptible individuals. Therefore, a note of caution is in order about the inclusion of large amounts of shrimp and

probably other crustaceans with a high cholesterol content in anti-atherogenic human diets until further studies in man clarify this problem.

The restriction of shrimp in the human diet because of its high cholesterol content is supported by recent studies from several different investigators, which emphasize the importance of dietary cholesterol in man. This substance has long been known as the most important single agent for the production of atherosclerosis in animals (Katz and Stamler, '53). Because cholesterol in the human diet appeared not to influence the serum lipids in earlier studies (Keys et al., '56), this dietary constituent was not believed to have pertinence to human atherosclerosis. Further investigations of this problem have demonstrated that dietary cholesterol has a distinct effect upon the serum lipids in man (Beveridge et al., '59a, '60b; Connor et al., '61a, b; Bronte-Stewart, '61; Steiner et al., '62).

The accumulation of cholesterol in the aortae of shrimp-fed rabbits paralleled closely the extent of gross atherosclerotic lesions. The rate of cholesterol accumulation varied greatly among rabbits. It was calculated that rabbit 6, a high responder, had an average daily deposition of 0.78 mg/gm of dried aorta. This was 60 times greater than the deposition of the aorta of rabbit 10, a low responder. Even in rabbit 6, however, the daily deposition of cholesterol was only a small fraction (roughly 0.2%) of the amount fed each day. It appears that the steady accumulation of small amounts of cholesterol in the arterial intima over a continuing time-span leads ultimately to advanced lesions.

The wide variation in the amount of atherosclerosis which the rabbits in this study manifested suggested the possible genetic nature of susceptibility to the dietary induction of this disease. Some rabbits (nos. 5 and 10) were resistant, whereas all of the other rabbits developed marked hypercholesterolemia and extensive atherosclerosis. Others have shown previously that rabbits have a wide variability in their hypercholesterolemic responses to cholesterol feeding (Fillios and Mann, '56). Physicians have long recognized that atherosclerosis is a familial disease in man. Families with similar living and eating patterns might have great variations in atherosclerotic disease. These differences in susceptibility for both animals and humans emphasize the need for studies to delineate the responsible mechanisms. Such studies might include differences in cholesterol absorption from the gut and in the elimination of cholesterol from the body through conversion to bile acids.

SUMMARY

A diet supplemented with shrimp was fed to rabbits for 24 weeks. These animals promptly developed a sustained hypercholesterolemia, averaging 795 mg/100 ml at the conclusion of the study. At autopsy, all rabibts had evidence of aortic atherosclerosis. In most, the process involved large areas of the intimal surface. The analysis of aortae for cholesterol content provided a more quantitative expression of the presence of atherosclerosis. Aortic cholesterol in the shrimp-fed rabbits was as much as 60 times greater than that in control rabbits.

Shrimp and other crustaceans contain large amounts of cholesterol. The results of this study re-emphasize that cholesterol-rich foods produce experimental atherosclerosis. Recent work has shown that dietary cholesterol has an important influence upon human serum lipid levels. Thus diets designed to prevent or treat human atherosclerosis probably should be restricted in shrimp and other sea foods high in cholesterol content.

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Distribution of the Bound Form of Nicotinic Acid in Natural Materials[1]

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The existence of an alkali-labile bound form of nicotinic acid, different from other known derivatives of nicotinic acid in cereal grains, has been established (Kodicek, '40; Krehl and Strong, '44). The bound form of nicotinic acid (called niacinogen) was first isolated from rice bran (Guha and Das, '57) and subsequently from wheat bran and whole corn (Das and Guha, '60). Kodicek and Wilson ('60) also reported the isolation of another bound form of nicotinic acid, called "niacytin," from wheat bran. It has been found that the bound form of nicotinic acid is unavailable or partly available to microorganisms requiring nicotinic acid for their growth (Krehl and Strong, '44; Kodicek and Pepper, '48; Das and Guha, '60), to rats (Chaudhuri and Kodicek, '60), to pigs (Kodicek et al., '59) and to man (Goldsmith et al., '56), unless the bound nicotinic acid is previously hydrolyzed with dilute alkali. That nicotinic acid could not be released from the bound form by pepsin (Das and Guha, '60) further indicates that bound nicotinic acid ingested through food would not be hydrolyzed by gastric juice. It appears, therefore, that the nicotinic acid as well as the nicotinamide content and not the bound nicotinic acid content of a foodstuff would determine its ability to cure or improve the nicotinic acid deficiency in animals. The present investigation was carried out to study the relative distribution of bound nicotinic acid, nicotinic acid and nicotinamide in a variety of foodstuffs in order to assess their nutritionally available nicotinic acid contents. In view of the absence of a suitable method, a microbiological method was also developed for the differential determination of nicotinic acid, bound nicotinic acid and nicotinamide in natural materials.

EXPERIMENTAL

The finely ground or homogenized sample (10 to 20 gm) was extracted with 5 to 10 times its weight of 0.1 N hydrochloric acid in a boiling water bath for 45 minutes. The extract was then cooled and centrifuged. The residue was treated twice with 5 to 10 ml of 0.1 N hydrochloric acid in the cold and then centrifuged. The combined centrifugate was used for the assay of bound nicotinic acid, nicotinic acid and nicotinamide.

The nicotinic acid content of each sample was determined microbiologically using *Leuconostoc mesenteroides* A.T.C.C. no. 9135 as the test organism which can utilize only nicotinic acid but can not utilize nicotinamide or bound nicotinic acid (Johnson, '45; Krehl et al., '46). The procedure of Johnson ('45) was followed except for the estimation of free nicotinic acid. The basal media was, however, modified according to Krehl et al. ('46).

The nicotinic acid (free) content was estimated from the extract without any hydrolysis of the bound form and the nicotinamide. Next the nicotinic acid plus the bound nicotinic acid content was determined by hydrolyzing the bound nicotinic acid with dilute alkali. The difference between the nicotinic acid content of the extract before and after alkaline hydrolysis represented the bound nicotinic acid content of the sample. Finally, bound nicotinic acid and nicotinamide were both hydrolyzed to nicotinic acid and

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the total nicotinic acid content was estimated. The difference between the total nicotinic acid content and the nicotinic acid content obtained after alkaline hydrolysis represented the nicotinamide content of the sample.

Estimation of nicotinic acid (free). An aliquot of the extract was taken and the pH was adjusted to 6.2 to 6.4. The precipitated materials were centrifuged off and the centrifugate was brought to pH 3.5 to 4. It was then sterilized by steaming at atmospheric pressure for 15 minutes. The solution was allowed to cool and the pH was then aseptically adjusted to 6.2 to 6.4 with a known quantity of sterile alkali. Aliquots of this solution were then aseptically added to tubes of suitably diluted and sterilized basal media. It was found that a portion of the bound nicotinic acid was hydrolyzed when the materials were sterilized at 10 p.s.i. for 15 minutes at pH 6.2 to 6.4, but when the sterilization was effected at pH 3.5 to 4 by steaming, only traces of nicotinic acid were released from the bound form.

Estimation of bound nicotinic acid. Bound nicotinic acid, together with nicotinic acid, was determined by hydrolyzing the bound form in the extract with 0.5 N sodium hydroxide for 10 minutes at room temperature. The pH of the solution was then adjusted to 6.2 to 6.4 and the insoluble materials removed by centrifuga-

TABLE 1

Distribution of bound nicotinic acid (BNA), nicotinic acid (NA) and nicotinamide (NAm) in natural materials[1]

	NA	BNA	NAm
	μg/gm	*μgNA/gm*	*μg/gm*
Rice[2] (*Oryza sativa*) Aman var. Latisail	8.4 ± 0.8[3]	53.2 ± 2.1	6.1 ± 0.3
Rice[2] (*Oryza sativa*) Aus var. Dular	9.1 ± 0.6	37.6 ± 3.1	4.7 ± 0.2
Rice[2] (*Oryza sativa*) Boro var. C.B. 1	6.2 ± 0.4	29.7 ± 2.2	2.8 ± 0.2
Wheat[2] (*Triticum sativum*) N.P. 798	3.8 ± 0.3	47.6 ± 3.5	4.2 ± 0.3
Wheat[2] (*Triticum sativum*) N.P. 826	4.5 ± 0.1	51.5 ± 2.1	5.2 ± 0.2
Maize[2] (*Zea mays*) V.L. 52	1.7[4]	34.5 ± 3.1	2.1 ± 0.2
Barley[2] (*Hordeum vulgare*)	10.2 ± 1.2	50.4 ± 4.5	5.1 ± 0.8
Jowar[2] (*Sorghum vulgare*)	2.8 ± 1.1	18.6 ± 3.2	0.8[4]
Bajra[2] (*Pennisetum typhoides*)	0.8[4]	18.1 ± 1.1	1.1[4]
Pea[2] (*Pisus sativum*)	14.2 ± 3.1	Nil	3.5 ± 1.1
Mung[2] (*Phaseolus aureus*)	10.1 ± 2.8	Nil	5.3 ± 2.1
Gram[2] (*Cicer arietienum*)	6.2 ± 0.8	Nil	12.1 ± 1.1
Groundnut[2] (*Arachis hypogea*)	89.3 ± 5.6	74.9 ± 10.1	10.1 ± 1.8
Mustard[2] (*Brassica campestris*)	42.3 ± 2.1	22.1 ± 2.2	8.1 ± 0.8
Active dried yeast[5]	301.2 ± 15.2	Nil	87.4 ± 10.2
Food yeast[6]	321.3 ± 10.5	Nil	93.9 ± 10.8
Prawn[7] (*Palaemon*) Galda	25.7 ± 5.2	Nil	13.0 ± 3.8
Prawn[7] (*Palaemon*) Bagda	26.4 ± 4.8	Nil	12.9 ± 4.1
Crab[7] (*Cancer*)	9.8 ± 2.2	Nil	15.0 ± 3.3
Oyster[7] (*Unio*)	8.3 ± 2.8	Nil	2.3 ± 1.1
Snail[7] (*Helix*)	13.6 ± 4.2	Nil	3.5 ± 1.4
Fish muscle[7] (Rohu)	2.5 ± 1.2	Nil	23.1 ± 4.8
Goat muscle[7]	5.5 ± 1.6	Nil	38.6 ± 3.5
Goat liver[7]	6.3 ± 1.9	Nil	145.7 ± 8.4
Goat blood[7]	1.2[4]	Nil	11.6 ± 3.7
Skim milk powder	0.4[4]	Nil	8.4 ± 1.7

[1] The values represent mean of three analyses and are expressed in terms of micrograms of nicotinic acid per gm of fresh tissue or per ml of whole blood.
[2] Purebred strains of the air-dried seeds were obtained from the Economic Botany Section of Government of West Bengal.
[3] SD from the mean value.
[4] Amount too small to be determined accurately.
[5] Central Food Technological Research Institute, Mysore, India.
[6] Koninkluke Nederlandsche Gist En., Holland.
[7] Obtained fresh from the local market.

tion. The nicotinic acid content of this solution was then determined.

Estimation of nicotinamide. Nicotinamide together with nicotinic acid and bound nicotinic acid was estimated by hydrolyzing the bound form with 0.5 N sodium hydroxide and then hydrolyzing the amide with 1 N hydrochloric acid at 15 p.s.i. for 1 hour (Krehl et al., '46). The pH of the solution was then adjusted to 6.2 to 6.4 and the nicotinic acid content of the solution was determined after centrifuging off the precipitate.

RESULTS AND DISCUSSION

The distribution of the bound nicotinic acid, nicotinic acid and nicotinamide is shown in table 1.

The results show that cereals contain 85 to 90% of their total nicotinic acid in the bound form. Pulses, however, do not contain any bound nicotinic acid. But oilseeds contain about 40% of their total nicotinic acid in the bound form. Bound nicotinic acid could not be found in yeast, crustacea, fish, animal tissues and milk. The distribution of nicotinamide shows that seeds contain only 5 to 10% of the total nicotinic acid as the amide but animal tissues contain 25 to 85% of their nicotinic acid as amide. Krehl et al. ('46) have also reported that the animal tissues contain most of their nicotinic acid in the amide form.

SUMMARY

The relative distribution of bound nicotinic acid, nicotinic acid and nicotinamide in a number of natural products has been surveyed microbiologically using *Leuconostoc mesenteroides* A.T.C.C. no. 9135 as the test organism. It appears from the results that most of the nicotinic acid in cereals and about 40% of the total nicotinic acid in oilseeds is present in bound form. Pulses do not contain any bound nicotinic acid, but contain only nicotinic acid and nicotinamide. Yeast, crustacea, fish, animal tissues and milk contain their nicotinic acid partly in the free form and mostly as the amide and they are devoid of any bound nicotinic acid.

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Macro- and Micromethods for the Determination of Serum Vitamin A using Trifluoroacetic Acid[1]

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The main problems encountered in the use of the Carr-Price (antimony trichloride) method for the determination of vitamin A have been well known since its original description (Carr and Price, '26). The antimony trichloride-chloroform reagent develops turbidity in the presence of trace amounts of moisture and the characteristic blue color formed by this reagent with vitamin A is subject to rapid fading. Despite these drawbacks, the Carr-Price reaction remains the method of choice for serum vitamin A determinations when sufficient blood can be obtained. On the micro-scale, however, the turbidity problem becomes unmanageable and for this reason Bessey et al. ('46) devised a microspectrophotometric method that has been widely employed. Dugan and Frigerio[2] have recently investigated the reactions of various Lewis acids with vitamin A and its derivatives. They found that trifluoroacetic acid (TFA) retained the sensitivity and specificity of the $SbCl_3$ reaction but did not exhibit the turbidity and film-forming properties of the latter reagent in the presence of moisture. This property suggested to us that TFA might not only be used to replace $SbCl_3$ in the conventional macro-determination of serum vitamin A but might also serve as the basis for a new simple micro technique. This paper reports the details of new macro- and micro- vitamin A procedures developed with the use of TFA as the chromogenic agent that offer certain advantages over methods now used.

MATERIALS AND METHODS

Reagents

Ethanol, 95%. Reagent grade was used without further purification.

Petroleum ether. Bottled, reagent grade benzine, boiling over a range of 38 to 56.9°C, was used without further purification for the micro- procedure. A special reagent grade petroleum ether,[3] boiling over a range of 30 to 40°C, was used without further purification for the macro-procedure.

Carr-Price ($SbCl_3$) reagent. A 20% solution of $SbCl_3$ in reagent grade chloroform was filtered repeatedly through anhydrous Na_2SO_4 until clear and was then stored in a brown bottle over anhydrous Na_2SO_4.

Trifluoroacetic acid (TFA) reagent. One volume of trifluoroacetic acid[4] was mixed with 2 volumes of reagent grade chloroform just prior to use. This solution is stable for 4 hours.

Acetic anhydride. Reagent grade was used.

Saponification mixture. Ninety per cent ethanol was made 1.0 N in KOH by the addition of an appropriate weight of KOH pellets.

β-Carotene standard solution. β-Carotene stored under nitrogen was used as a standard.[5] A 50-mg sample of this was dissolved in a few milliliters of chloroform and brought to a final volume of 100 ml with petroleum ether. One milliliter of this solution was then diluted to 100 ml with petroleum ether to form an intermediate standard. This solution is stable for only a few hours and should be made up just prior to use.

Vitamin A standard solution. The USP reference standard solution of vitamin A

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[2] Dugan, R. E., and N. A. Frigerio 1961 A.C.S. Abstracts, 160: 65c.

[3] Ligroine, Baker Chemical Company, Phillipsburg, New Jersey.

[4] Eastman Organic Chemicals no. 6287.

[5] Nutritional Biochemicals Corporation, Cleveland.

acetate in cottonseed oil[6] was used. This standard contains 34.4 mg of all *trans* vitamin A acetate/gm of solution. A 100-mg sample of this standard (containing 3.44 mg of vitamin A acetate) was dissolved in chloroform and diluted to 50 ml in a volumetric flask. This solution is stable for at least two days when refrigerated and kept protected from light.

Apparatus

A Coleman Jr. Spectrophotometer equipped with 10 × 75 mm cuvettes was used for the macro- analyses. The micro-analyses were carried out in a Beckman model DU spectrophotometer equipped with the micro- attachments described by Lowry and Bessey ('46). Microcuvettes measuring 2.5 × 10 × 25 mm were used.[7] For the micromethod extractions a special mixer[8] was used. The high-speed drill mixing device described by Bessey et al. ('46) also proved to be satisfactory. Lang-Levy constriction pipettes of 0.01-, 0.05-, 0.075-, and 0.100-ml capacities were used in the micromethod. Two-tenths-milliliter serological pipettes were drawn out to slender tips for use in pipetting petroleum ether in the micro- procedure.

Material for analysis

Human plasma for analysis was obtained from the Vanderbilt clinical laboratory or blood bank. It was usually necessary to pool plasma samples from the clinical laboratory to obtain enough for replicate analysis. Heparinized rat blood was obtained by heart puncture. Porcine and bovine blood was taken from individual animals at a local abbatoir. In this instance, serum was used. All samples were kept frozen until analyzed.

Macro- procedure

This procedure is basically a modification of the methods of Kimble ('39) and Kaser and Stekol ('43) except that TFA is substituted for $SbCl_3$.

Transfer in duplicate 2 ml of serum into 16 × 125 mm glass stoppered test tubes. Add, with mixing, 2 ml of 95% ethanol followed by 3.0 ml of petroleum ether (boiling range 30 to 40°C). Stopper and shake vigorously for two minutes to insure complete extraction of carotene and vitamin A. Centrifuge the tubes slowly for three minutes. Carefully pipette off 2.0 ml of the petroleum ether (upper) layer and place it in a Coleman 75 × 100 mm cuvette. Stopper immediately with a cork and read the carotene at 450 mμ against a petroleum ether blank in the Coleman Jr. spectrophotometer. Remove the cuvette and evaporate the petroleum ether to dryness in a 35 to 40°C water bath. An impinging stream of nitrogen may be used to increase the rate of evaporation but this is not necessary. Take up the residue immediately in 0.1 ml of chloroform and add 0.1 ml of acetic anhydride. Set the Coleman spectrophotometer at 620 mμ to zero optical density with a blank consisting of 0.1 ml of chloroform and 1.0 ml of TFA reagent. Place the sample cuvette in the spectrophotometer, add 1.0 ml of TFA reagent and record the reading at the pause point or alternately, exactly 30 seconds after addition of the reagent. The former may be defined as the maximal "hesitation" reading that occurs after the galvanometer has recovered from its initial swing. The timed reading is to be preferred.

Standard curves and calculations

The β-carotene intermediate standard is diluted with petroleum ether to give solutions containing 0.5, 1.0, 1.5, and 2.0 μg of β-carotene/ml, respectively. The optical densities of these solutions are read at 450 mμ against a petroleum ether blank and a standard curve plotted. It should be mentioned that β-carotene is a notoriously labile compound even when extreme care is taken in its storage. Under our laboratory conditions, F = 6.8 in the following equation:

$$F = \frac{\mu g \text{ carotene/ml}}{\text{optical density}}$$

Although it might be expected that this factor would vary slightly from laboratory to laboratory, any gross deviation should be cause to suspect the purity of the standard.

Since β-carotene also reacts with the TFA reagent to give a blue color, it is necessary to run a TFA — carotene stand-

[6] U.S. Pharmacopoeial Convention, 46 Park Avenue, New York 16, New York.
[7] Pyrocell Manufacturing Company, New York 28, New York.
[8] Cyclo-Mixer, Clay-Adams, Inc., New York 10, New York.

ard curve to permit calculation of a correction factor. Make up carotene standards in chloroform for this determination to contain 4.0, 8.0, and 10.0 μg/ml. Place 0.1-ml aliquots in Coleman cuvettes, and carry out the TFA reaction as previously described. Under our laboratory conditions:

$$OD_{450} \times 0.300 = OD_{620}$$

Vitamin A standards are prepared from the stock standard solution to give solutions containing 6.8, 13.7, 20.6 and 34.4 μg/ml, respectively. For preparation of the standard curve, pipette 0.1-ml aliquots of these standards into Coleman cuvettes for reaction with the TFA reagent. Under our laboratory conditions F = 4.49 in the following equation:

$$F = \frac{\mu g \text{ vitamin A/tube}}{\text{optical density}}$$

From the foregoing calculations and the volumes of reagents used, the amounts of β-carotene and vitamin A in the sample are calculated as

(1) carotene

$OD_{450} \times 1020 =$ μg carotene/100 ml serum

(2) vitamin A

$OD_{620} - (OD_{450} \times 0.300) \times 337 =$ μg vitamin A/100 ml serum.

The factors shown in the above equations should be derived by each operator, particularly in view of the fact that a new and relatively untested chromogen has been introduced. We have, however, found no differences in TFA — vitamin A color yields with 14 separate bottles of this reagent purchased at different times.

Micro- procedure

The micromethod is essentially the same as the macromethod except that it has been adapted to a much smaller scale. Pipette either 0.1 or 0.05 ml of serum into a 6 × 50 mm test tube. Add an equal volume of 95% ethanol (Lang-Levy pipette) and 0.15 ml of petroleum ether (38 to 56.9°C). Stopper the tube immediately with a cork and extract the vitamin A and carotene by agitation with the special mixer[9] for 45 seconds. Centrifuge the tube for 10 minutes at 3,000 × *g* and transfer 0.10 ml of the petroleum ether layer to a micro-cuvette by means of a Lang-Levy fine-tipped constriction pipette. Read the carotene optical density immediately at 450 mμ against a petroleum ether blank. Transfer as much of the sample as possible to a clean 6 × 50 mm test tube and rinse the cuvette once with 0.05 ml of petroleum ether. Add the rinsing to the sample in the test tube. Evaporate to dryness in a 40 to 50°C water bath (5 minutes). Take up the residue in 0.01 ml of chloroform and add 0.10 ml of the TFA reagent with vigorous shaking. Transfer rapidly as much of the solution as possible to the micro-cuvette by means of a 0.10-ml Lang-Levy pipette. Take the vitamin A reading at 620 mμ against a TFA reagent blank exactly 30 seconds after the addition of the TFA reagent. It is essential that this step be carried out with great care, particularly with respect to the timing because the color fades.

Carotene and vitamin A standard curves are prepared using smaller aliquots of the same standards used in the macro- procedure.

RESULTS

Trifluoroacetic acid — chloroform mixtures were tested for vitamin A color yields in the proportions of 1:1, 1:2, 1:5, 1:10 and 1:20. The 1:2 and 1:5 mixtures gave about the same color yields but the other mixtures gave considerably less. The 1:2 mixture was used throughout this study.

The blue species produced by the interaction of TFA, and vitamin A in chloroform was found to exhibit maximal absorption of 616 mμ in accordance with the observations of Dugan.[10] After standing for about two hours the original blue color decayed to a pink with a maximal absorption at about 540 mμ but of greatly reduced sensitivity. No attempts were made to use this color analytically. Various fat-soluble substances (cholesterol, calciferol, vitamin E, vitamin K) gave no color with TFA when tested at concentrations greater than those normally found in sera. An impure sample of xanthophyl gave virtually no color at concentrations deemed to lie within the physiological range.

[9] See footnote 8.

[10] Dugan, R. E., 1962, personal communication.

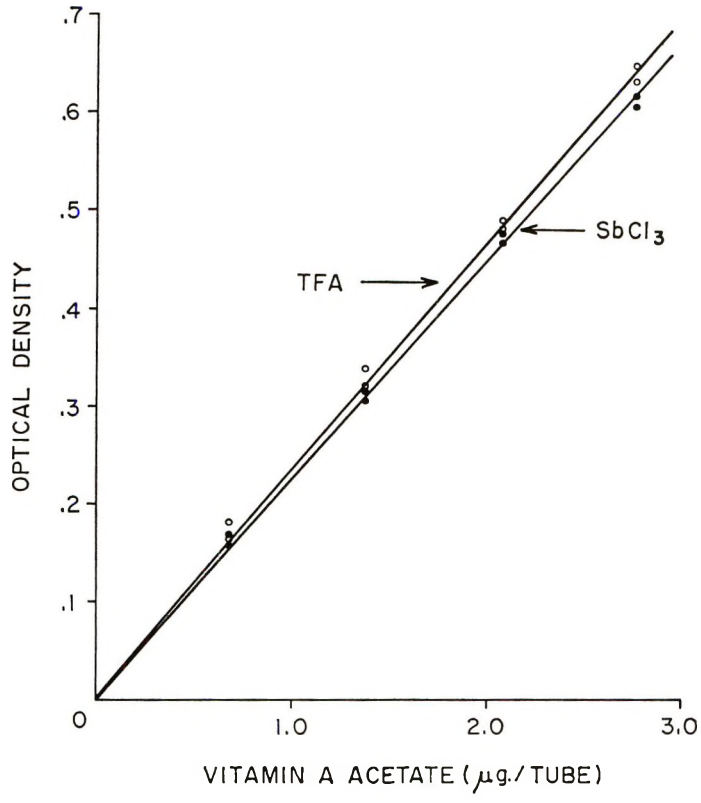

Fig. 1 Comparison of vitamin A standard curves obtained with antimony trichloride and trifluoroacetic acid. Readings were made in 75 × 100-mm cuvettes with a Coleman Jr. spectrophotometer at 620 mμ.

Typical vitamin A acetate dose-response curves measured at 620 mμ in the Coleman Jr. spectrophotometer with TFA or $SbCl_3$ are shown in figure 1. The color yield with TFA was also slightly greater than that with $SbCl_3$ with vitamin A alcohol and vitamin A acid. The yield of colored complex formed with vitamin A acid was less than half that with vitamin A acetate. This vitamin A acid — TFA complex formed slowly and was a deep purple rather than a typical Carr-Price blue. β-Carotene gave less than one-tenth the color yield of vitamin A at 620 mμ with both reagents. The carotene color yield with TFA was slightly less than that with $SbCl_3$.

The stability of the colored species formed with TFA varied depending upon the type of cuvette used. Representative time-decay curves with vitamin A acetate and serum extracts are shown in figure 2. In a micro-cuvette the color formed with a vitamin A acetate standard faded about 12% by 1 to 1.5 minutes after the initial reading, whereas the same reaction in a Coleman cuvette faded by approximately 20%. Under the same conditions the colored species formed with a serum extract faded 10% in a micro-cuvette as opposed to 25% in a Coleman cuvette.

The TFA method was compared with the classic $SbCl_3$ technique using human, rat, bovine and porcine sera (table 1). The two methods agreed quite well when human, rat, or porcine sera were analyzed. Generally TFA values were slightly higher. When bovine sera containing large amounts of carotene were analyzed, however, the vitamin A values obtained with TFA were markedly higher than those obtained with $SbCl_3$. When the carotene values were low (bovine samples 8 and 9), good agreement was obtained. This

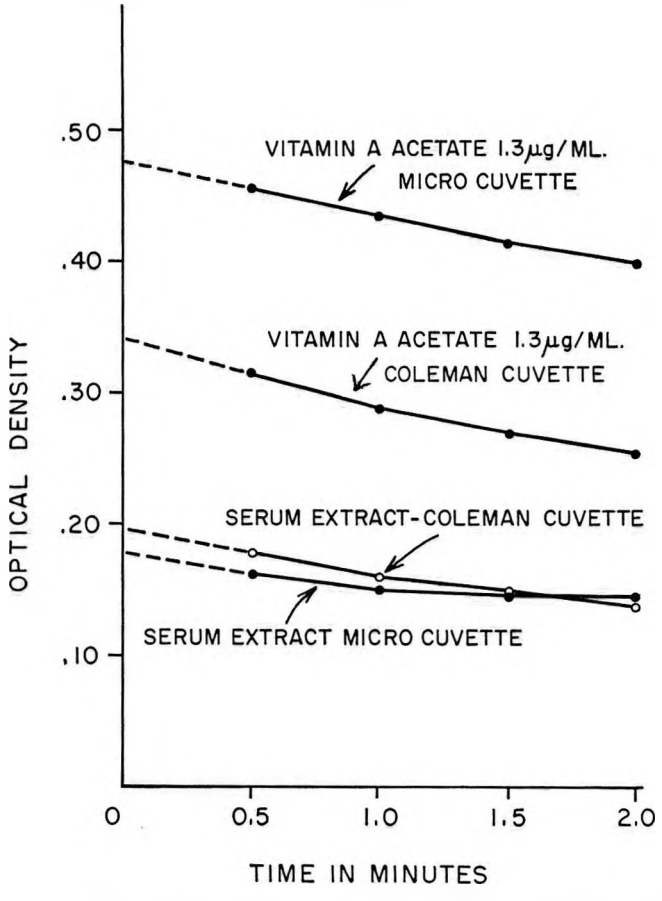

Fig. 2 Trifluoroacetic acid — vitamin A fading curves. Initial readings were taken exactly 30 seconds after addition of the TFA and then at the time intervals and in the instrument specified.

implies that the two chromogens react differently with the carotenoids or the other pigments normally present in bovine sera, or with both. Since serum carotene levels of 300 μg/100 ml are but rarely encountered in the human, this does not militate against the routine use of TFA for the analysis of human sera.

Reproducibility studies were carried out with $SbCl_3$ and TFA by analyzing the same serum sample repeatedly both on the same day and on different days (table 2). In both situations TFA performed slightly better than did $SbCl_3$. This may be a function of the $SbCl_3$ moisture problem since amounts of turbidity, sufficient to affect the optical density readings, often escape the analyst's eye and might therefore result in greater variability.

Table 3 presents a comparison of vitamin A values determined by the usual procedure or after a preliminary saponification of the serum. The vitamin A values obtained after saponification were slightly higher than those obtained without saponification, but carotene levels after saponification were distinctly lower in several samples. Our results suggest, therefore, that there is some advantage to be gained by saponification, which is in accordance with the report of Bessey and Lowry ('46).

Recovery experiments were performed on a series of human sera using both the $SbCl_3$ and TFA methods. These were determined in triplicate by comparing the increase in Carr-Price color resulting from the addition of vitamin A acetate to sam-

TABLE 1

A comparison of $SbCl_3$ and trifluoroacetic acid (TFA) plasma vitamin A values in various species

Sample	Vitamin A		TFA
	Carotene	Sb Cl_3	
	μg/100 ml	*μg/100 ml*	
	Bovine[1]		
1	848.6	40.9	55.2
2	823.4	38.9	56.1
3	476.3	21.6	35.2
4	744.5	36.9	49.9
5	722.4	27.3	41.1
6	772.9	31.9	36.8
7	1116.8	118.5	129.1
8	93.3	19.9	21.5
9	47.9	20.6	20.9
Mean	627.3	39.6	49.5
SD[2]	356.1	30.6	32.5
	Rat		
1	5.6	30.3	31.5
2	0.0	48.6	46.4
3	1.8	25.9	24.9
4	12.5	38.6	43.0
Mean	4.9	35.8	36.4
SD	5.5	9.9	9.9
	Human		
1	115.0	38.4	39.2
2	65.5	25.8	32.2
3	106.3	26.6	28.0
4	136.4	42.0	47.5
5	71.6	39.7	39.3
6	63.5	37.2	39.4
7	116.5	49.7	52.3
8	180.6	35.1	36.3
9	148.6	36.5	40.3
10	74.8	39.2	47.8
Mean	107.8	37.0	40.2
SD	39.5	7.0	7.4
	Porcine[1]		
1	4.4	26.6	30.2
2	2.5	29.3	31.2
3	6.9	39.6	38.6
4	1.8	19.9	21.8
5	0.0	25.9	26.8
6	0.0	24.6	27.4
7	0.0	29.6	34.6
8	3.7	21.9	23.0
9	1.2	13.3	16.2
Mean	2.3	25.6	27.7
SD	2.3	7.3	6.8

[1] Serum.
[2] Standard deviation.

ples of known vitamin A content. The data in table 4 show that both methods gave good recoveries of the added vitamin A acetate.

In table 5 representative results obtained with the TFA macro- and micromethods are compared. The agreement of values was relatively good except in sample 24 which contained an unusually large amount of carotene.

Essentially the same values were obtained when 0.05 and 0.10 ml of serum were analyzed by the micromethod (table 6). In these analyses each serum was analyzed in triplicate at the two volume levels. The amount of alcohol added was equal to that of the serum but the same amount of petroleum ether (0.15 ml) was used for extraction.

DISCUSSION

The color reaction of TFA with vitamin A appears to be suitable for the measurement of vitamin A in serum since the values obtained in human, rat, and porcine sera are similar to those found with the Carr-Price reaction. The direct measurement of vitamin A with TFA in bovine sera without the prior removal of carotene requires further study before its validity can be assessed.

The use of TFA eliminates entirely the moisture problem encountered with $SbCl_3$. The TFA is also less toxic than $SbCl_3$ and unlike the latter does not form a tenacious film on the cuvette.

The main drawbacks of the TFA method are the evanescent nature of the blue color and the extreme volatility of the solvents used. The former has been satisfactorily controlled by reading exactly 30 seconds after the addition of TFA to the serum extract. The low boiling range petroleum ether used in the macromethod was entirely too volatile for the micromethod but the higher boiling petroleum ether product (40 to 60°C) proved to be acceptable if the various manipulations were performed rapidly. Ambient temperature in our laboratory averaged 21 to 23°C during these studies. Hexane was also found to be a suitable extractant and possessed the advantage of being considerably less volatile than petroleum ether. It would undoubtedly be a more desirable solvent for use at ambient temperatures higher than ours. Evaporation of a hexane extract in a 40°C water bath took an unduly long period of time, but an impinging stream of nitrogen from a hypodermic

TABLE 2

Reproducibility of $SbCl_3$ and trifluoroacetic acid (TFA) values for human serum vitamin A[1]

Experiment A			Experiment B		
Sample	$SbCl_3$	TFA	Sample	$SbCl_3$	TFA
	μg/100 ml			*μg/100 ml*	
1	47.6	54.9	11	48.6	45.2
2	48.6	51.4	12	48.9	53.9
3	50.9	48.3	13	49.6	51.1
4	48.9	49.6	14	45.2	51.7
5	51.2	49.2	15	46.6	49.6
6	50.2	50.2	16	50.2	52.1
7	50.6	48.9	17	41.6	50.5
8	48.2	49.2	18	43.9	52.1
9	44.9	48.6	19	40.9	53.6
10	51.2	48.6	20	42.6	51.7
Mean	49.2	49.8	Mean	45.8	51.1
SD[2]	2.0	1.9	SD	3.4	2.4
C[3]	4.0%	3.8%	C	7.4%	4.6%

[1] In experiment A, 10 aliquots of a pooled serum sample were analyzed simultaneously. In experiment B, an aliquot of the same pooled sample was analyzed daily for 10 consecutive days.
[2] Standard deviation.
[3] Coefficient of variation.

TABLE 3

Effect of saponification upon vitamin A values of human serum[1]

Sample	Saponified			Unsaponified		
	Carotene	Vitamin A		Carotene	Vitamin A	
		$SbCl_3$	TFA		$SbCl_3$	TFA
	μg/100 ml	*μg/100 ml*		*μg/100 ml*	*μg/100 ml*	
11	76.7	53.9	51.5	89.9	56.1	50.2
12	73.5	39.6	37.4	63.5	37.2	39.4
13	154.7		37.3	180.6		36.5
14	125.3		49.3	148.6		40.3
15	75.6		50.2	74.8		47.8

[1] Saponification was carried out at 60° for 20 minutes with 1 N alcoholic KOH.

TABLE 4

Vitamin A recovery from serum samples

Sample	Vitamin A content of serum	Vitamin A acetate added	Expected vitamin A	Actual vitamin A	Recovery
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	*μg/100 ml*	%
			$SbCl_3$		
16	49.5	51.6	101.1	93.7	92.6
17	25.8	51.6	77.4	72.7	93.9
18	49.4	51.6	101.0	98.7	97.7
19	26.6	41.2	67.8	72.0	106.1
20	42.0	41.2	83.2	84.3	101.3
			Trifluoroacetic acid (TFA)		
16	50.4	51.6	102.0	99.6	97.6
17	32.2	51.6	83.8	82.1	97.9
18	55.5	51.6	107.1	99.9	93.2
19	28.0	48.6	76.6	73.1	95.4
20	47.5	48.6	96.1	92.7	96.4

TABLE 5

Comparison of macro- and micromethods for vitamin A analysis using trifluoroacetic acid (TFA)

Sample	Macromethod		Micromethod	
	Carotene	Vitamin A	Carotene	Vitamin A
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	*μg/100 ml*
21	109.3	44.0	112.3	42.4
22	138.7	24.4	140.8	28.3
23	116.2	40.7	113.0	41.1
24	283.0	28.2	264.4	36.9
25	202.4	35.7	191.7	41.3

TABLE 6

Comparison of vitamin A levels obtained with different amounts of serum

Sample	0.050 ml serum		0.100 ml serum	
	Carotene	Vitamin A	Carotene	Vitamin A
	μg/100 ml	*μg/100 ml*	*μg/100 ml*	*μg/100 ml*
26	79.9	39.1	107.8	41.1
27	122.3	50.2	129.0	46.1
28	107.2	43.7	118.1	42.1
29	145.4	38.2	171.1	32.1

needle took it to dryness in about one minute. Heptane, benzene, and xylene were suitable extractants, but their higher boiling points made them not useful. Although much has been made of the possibility of oxidative loss of vitamin A during the evaporation step, we have been unable to demonstrate such a loss at 40°C even by the use of an impinging stream of air.

In our laboratory, saponification resulted in a slightly higher vitamin A value although not of the magnitude reported by Bessey et al. ('46) and Sobel and Snow ('47). It would therefore appear to be appropriate to carry out a saponification prior to extraction. Oser et al. ('43) recommend the use of an internal vitamin A standard to reduce the error introduced by the effect of inhibitory substances on color development. Bessey et al. ('46) found that vitamin A values were from 5 to 10% higher when calculated by this procedure than those taken from a conventional standard curve. Accordingly the use of an internal standard would increase somewhat the accuracy of the method, but in practice this approach would depend largely upon the availability of serum because duplicate samples are required. In nutrition surveys the usual quantity of blood available from finger-tip puncture normally would not be sufficient for this purpose.

Attempts to make the method more rapid by the direct addition of undiluted TFA to serum extracts were not successful. Although the TFA—vitamin A color formed readily with vitamin A standards in both hexane and benzene, some serum extracts gave no color with TFA and, for unknown reasons, TFA was insoluble in hexane extracts of sera. Whereas $CHCl_3$, CCl_4, and $C_2H_4Cl_2$ gave full color yields with vitamin A and TFA, they failed to extract quantitatively vitamin A and carotene from the serum.

The TFA method offers its greatest advantage over existing methods at the micro- level. The only micromethod in common use is that of Bessey et al. ('46), which depends upon the measurement of the ultraviolet absorption of serum extracts at 328 mμ before and after irradiation with ultraviolet light. The main source of difficulty with this technique is that ultraviolet light not only destroys vitamin A but changes other substances that absorb at 328 mμ as well. This may introduce a serious error, especially when low vitamin A levels are being measured (Caster and Mickelsen, '55). The observations of Bieri and Schultze ('51) and

Sobel and Snow ('47) that hemolysis results in consistently high values for vitamin A and carotene when measured by this method were confirmed by Utley et al. ('58). These investigators reported that a rigid purification of the kerosene-xylene extraction mixture was necessary to insure reliable results and concluded that the Carr-Price method was the method of choice when sufficient serum was available. In addition to these shortcomings, the Bessey method is tedious and time consuming and requires an ultraviolet spectrophotometer and a special irradiation device. The TFA micromethod described here is about three times more sensitive than the spectrophotometric procedure, does not require elaborate purification or drying of solvents, is not affected by hemolysis and does not require the use of an ultraviolet spectrophotometer. Although an ultraviolet spectrophotometer was used in this study, a few trials with the Beckman Spinco Model 151 microspectrophotometer[11] suggest that this instrument could also be used. The TFA method is considerably more rapid than the Bessey procedure. One operator can easily analyze 20 samples in duplicate in three hours. It is, moreover, even more rapid than the macro- procedure because the solvent evaporation time is reduced from ± 30 minutes (macro-) to ± 5 minutes (micro-).

SUMMARY

A new method for the determination of plasma or serum vitamin A levels which uses trifluoroacetic acid (TFA) as the chromogen is described. This reagent produces a typical Carr-Price color but does not exhibit the turbidity or film-forming properties of $SbCl_3$ in the presence of moisture. The new procedure gives values that agree well with those obtained by the Carr-Price method when rat, porcine, or human sera are analyzed. Significantly higher values are obtained with TFA, however, when bovine sera were analyzed. A micro- modification of the method permitting the analysis of 50 µl of serum is described and its advantages over existing methods are discussed.

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Urinary Creatinine as an Index of Body Composition[1]

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In accord with modern trends in human dietary regimens, greater emphasis is being given hopefully to the genetic selection of meat-producing livestock whose progeny will produce more protein and less fat. The potential success of such genetic improvement is enhanced by the recent more general application of artificial insemination in meat-producing species of farm animals. As a consequence, a simple and sufficiently precise method of predicting the lean-body mass or the total body protein of the living animal would be an important corollary tool for the genetic improvement of livestock, as well as serving to provide significant economic criteria. Similarly, such a method has a great potential value in nutritional and physiological investigations in man and other animals.

During the past 30 years the mounting interest in the chemical composition of the body of animals and man has resulted in the development of a number of techniques for estimating body composition in the living organism. In general these techniques make use of the fact that the major chemical constituents of the ingesta-free body are intimately related quantitatively and thus the quantity of certain constituents is predictable from that of others (Reid et al., '55; Clawson et al., '55). The lean-body mass can be estimated indirectly by means of various methods such as the dilution techniques of predicting either the water or the fat content of the body, but in the ruminant the application of such methods is complicated by the usually large, but variable, quantity of digestive-tract contents. However, the combined use of the dilution methods using antipyrine and N-acetyl, 4-aminoantipyrine as reference substances has been proposed for estimating the water content of the ingesta-free body of ruminants (Reid et al., '57). The use of this scheme to derive the ingesta-free body mass requires in addition some means of estimating the dry matter content of the ingesta.

Of the methods used to estimate lean-body mass, that employing the urinary creatinine output as the index has been one of the most generally used. This method is based on the assumption that the amount of creatinine excreted reflects the size of the muscle mass of the body. Although this postulate has been widely accepted and used during the past 55 years, it is based on little direct evidence.

Borsook and Dubnoff ('47) found that 98% of the creatine reserves of the body exist in the muscles, mainly in the form of phosphocreatine. They observed that approximately 2% of the creatine of the body is converted each day into creatinine which is excreted in the urine. Their analysis of the data presented by other workers indicated that in 4 animal species the amount of creatinine excreted in the urine per day is highly correlated with the amount of creatine in the body. More recently Kumar et al. ('59), as the result of making comparisons of body weight and urinary creatinine output with the fat-free mass of rats (determined by direct analysis), concluded that urinary creatinine contributed little additional information not provided by live weight to the prediction of the fat-free mass. Nevertheless, these workers observed a highly predictable relationship between urinary creatinine and the lean-body mass. Since the animals involved in that study were still growing and were of rather similar body composition (e.g., the fat content ranged from only 8.4 to 13.7%), it is

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axiomatic that body weight would be an accurate predictor of the lean-body mass. Thus, it is not expected that a consideration of creatinine output in addition to body weight would improve very much the prediction of lean-body mass.

The approach taken by other workers has been less direct, in that muscle mass or its equivalent has been estimated. For example, Lofgreen and Garrett ('54) correlated the amount of lean tissue separated manually from the fat and bone of the 9–10–11 rib cut of cattle with urinary creatinine excretion. Although these variables were significantly correlated ($r = 0.67$), the relationship had a low prediction value. The correlations between urinary creatinine output and the estimated muscle mass have in general supported the concept that these two variables are related, but they have been no better than those between estimated muscle mass and the live weight of the animal.

The objectives of the investigations to be reported here were: (1) to study the relationships between the amount of creatinine excreted in the urine and the quantities of the gross chemical components in the ingestra-free body of sheep; (2) to determine the accuracy with which certain of the major chemical constituents in the living animal can be predicted from the amount of urinary creatinine excreted; and (3) to examine the effects of level of feed intake and age of animals on the relation between urinary creatinine output and the chemical composition of the body.

EXPERIMENTAL PROCEDURE

Plan of experiment and animals used. The objectives of this study were accomplished by (1) measuring the urinary creatinine excretion rate during 7- to 10-day periods just prior to slaughter, and (2) measuring the proximate chemical constituents of the ingesta-free body. The data so obtained were submitted to analyses of correlation, regression and variance.

Sixty-five sheep ranging from 4 to 27 months of age at the time of slaughter were used in this study. Of these animals, 63 had been used in a nutritional experiment; 18 of these were slaughtered at the beginning of the experiment and, thus, were called the "initial" slaughter group, and the remaining 45 were slaughtered at the end and were called the "final" slaughter group. Two additional animals composed the "supplementary" slaughter group. These animals were of different ages and had been exposed to various nutritional treatments to effect a wide range in body composition; therefore, they can be described most conveniently as members of the following slaughter groups: (1) the "initial" slaughter group consisted of 18 sheep of which 9 were 8-month old Hampshire × Shropshire × Suffolk wether lambs and 9 were 20-month old wethers of Western origin; (2) the "final" slaughter group was made up of 45 sheep of which 18 were 15-month old Hampshire × Shropshire × Suffolk wethers and 27-month old Western wethers; and (3) the "supplementary" group consisted of one 4-month-old Dorset Horn wether lamb and one 27-month-old Shropshire × Hampshire wether.

Feeding treatments imposed. The nutritional history of each of the groups was as follows: (1) the sheep in the "initial" slaughter group were fed a hay ration ranging from a sub-maintenance to a maintenance level of intake for two months prior to slaughter in order to reduce the amount of body fat in each animal to about the same, low level; (2) the animals of both age groups represented in the "final" slaughter group were fed one of three kinds of feed (chopped, early-cut timothy hay; the same hay in pelleted, finely ground form; a pelleted mixture of 55% of the same finely ground hay and 45% of corn meal) at one of three levels of intake (as percentages of the feed intake maintaining energy equilibrium: 109, 137, 190) continuously for 196 days before slaughter; thus, the 45 animals of this group were used in a factorial experiment involving two ages × three kinds of feed × three levels of feed intake; and (3) of the animals of the "supplementary" group, the 4-month-old lamb had received a maintenance ration of hay for about one month after weaning, and the 27-month-old wether had received ad libitum a pelleted mixture of 45% corn meal and 55% of finely ground hay for over a year prior to slaughter.

Collection, sampling and creatinine analysis of urine. The total quantity of urine voided was collected from all sheep during 7- to 10-day periods immediately prior to slaughter. Sufficient 4 N H_2SO_4 was added to the urine at the time of each daily collection to reduce the pH to 2.5 to 3.5. Composite samples representing 10% aliquots of the urine produced each day were stored at 0° to 4° until analyzed according to the method proposed by Owen et al. ('54) as described by Van Niekerk et al. ('63). The output of creatinine during the 7- to 10-day collection periods was expressed in terms of milligrams per 24 hours for each animal.

It has been demonstrated that exogenous dietary creatinine, fed at a level several times as high as that normally present in the diet of herbivores, has no measurable effect on the rate of urinary creatinine excretion in sheep (Van Niekerk et al., '63). Thus, no correction for dietary creatinine was made in the present study. In previous experiments (Van Niekerk et al., '62), it was shown that the creatinine in sheep urine decays at a differential rate depending on the environmental temperature during the urine-collection period. Accordingly a correction was made for the quantity of creatinine lost during the urine-collection process using the equation reported previously (Van Niekerk et al., '63).

Slaughter procedure. The day before slaughtering each sheep was shorn closely. Although the wool was analyzed separately, its components were not included in computing the composition of the whole body. The sheep received water but were deprived of feed for 18 to 20 hours just prior to slaughter. The live-body weight was taken at the time of slaughter and the body of each animal was divided into the following "tissue" components: (1) blood, which was obtained by severing the major blood vessels in the neck; (2) hide minus the wool, but including the ears, hooves and pasterns; (3) the viscera minus the contents of the gastrointestinal tract; (4) one-half of the skinned, eviscerated carcass; and (5) the contents of the digestive tract.

The carcass (containing its normal complement of bones), empty viscera and hide were ground individually by a high-capacity grinder. The grinding process was repeated 5 to 6 times to insure the preparation of a representative sample and required only 3 to 4 minutes. The slaughter operations were performed as rapidly as possible with precautions being taken to avoid moisture losses. However, moisture lost during the preparation of analyzable samples was accounted for by observing weight at the various stages.

The fatter sheep of the "supplementary" group were ground as a single sample with only the contents of the gastrointestinal tract removed.

Drying, sub-sampling, and analysis of body components. The dry matter content of the gross tissue samples was determined by freeze drying two 700- to 1200-gm sub-samples of each tissue group. To reduce the particle size still further for the proximate analyses, the dried samples were ground in a Wiley laboratory mill equipped with a 1/16 inch screen. This was accomplished by freezing the bulked, dried samples with crushed dry-ice weighing approximately three times as much as the samples with which it was mixed. The finely ground samples were stored in a freezer for 24 hours to allow the dry-ice to sublimate and then sub-samples were taken and kept frozen until analyzed. Based upon the high repeatability of the analytical results observed in these studies, this procedure resulted in very homogenous samples.

Ash was determined by the usual AOAC ('55) method and nitrogen was determined by the Kjeldahl method, using the boric-acid modification suggested by Scales and Harrison ('20). Fat was recorded as the difference between the dried sample and the residue remaining after extraction with ether. Four to 8 samples weighing approximately 10 gm were extracted continuously for 7 days. The moisture content of the "dried" tissue samples was also determined by freeze drying.

RESULTS AND DISCUSSION

Body composition. Since the population of sheep used in this study represented a great range in age and nutritional history, it also represented a wide range in body size and composition. The live-

body weight of the 65 sheep ranged from 17.1 to 75.8 kg. It was observed that the contents of the digestive tract constituted a rather varible proportion of the live weight, but that the proportion of contents was influenced mainly by the size and degree of fatness of the animal. In the fattest sheep the ingesta constituted only 6% of the live weight, whereas in one of the thinnest animals the contents comprised 26% of the live weight. These proportions are expected to be somewhat less than those under normal conditions because these sheep had been fasted for 18 to 20 hours prior to slaughtering.

From the chemical analyses made of the individual "tissue" components, the amounts of moisture, protein, fat, and ash were determined in each of the tissue-component groups and, thus, also in the ingesta-free total body of each sheep. The average of, and the range in, the amounts of the proximate constituents and the fat-free mass in the 65 sheep are shown in table 1. The average of, and the range in, the percentages of the proximate constituents in the ingesta-free total body of the same sheep are recorded in table 2. These data show that not only did this population represent a wide range in total body constituents (which is largely the result of a great range in body size), but it also represented a wide range in the chemical composition expressed as a percentage of the body mass. The range of 4.86 to 46.60% of fat approximates the maximal range in the degree of fatness for the kinds of sheep used in this study. The fattest animal in this study appears to have contained even more fat than the famous "extra fat sheep" analyzed by Lawes and Gilbert (1859). Although the latter animal contained 45.8% of fat on the whole-body (ingesta included) basis, the wool fat was also included.

TABLE 1

Weights of constituents in total ingesta-free bodies of 65 sheep

Constituent	Average	Range
	gm	*gm*
Water	19,560	9,720 to 35,940
Dry matter	15,610	3,450 to 37,930
Fat	9,020	640 to 29,240
Protein[1]	5,200	2,080 to 9,450
Ash	1,240	580 to 2,410
Fat-free mass	26,140	12,520 to 48,160

[1] Protein calculated as 6.25 × nitrogen determined directly.

TABLE 2

Percentages of constituents in ingesta-free bodies of 65 sheep

Constituents	Average	Range
	%	%
In empty body:		
Water	58.25	39.60 to 73.82
Fat	22.36	4.86 to 46.60
Protein	15.23	10.67 to 17.72
Ash	3.75	1.72 to 5.56
Dry matter	41.75	26.18 to 60.40

Effect of diet on creatinine excretion. The data, obtained with the 45 sheep of the "final" slaughter group representing two age groups and three diets, each of which was fed at three levels of intake for 196 days, were examined as a 2 × 3 × 3 factorial experiment to determine the effects of age of sheep, and kind and amount of diet on the rate of creatinine excretion. An analysis of variance revealed no statistically significant differences between ages (15 and 27 months), among diets (chopped hay, pelleted hay, and pelleted mixture of corn meal and ground hay), or among levels of intake (1.09, 1.37, and 1.90 times maintenance) in the amount of urinary creatinine excreted per day per unit of body protein. Also, there were no mathematically significant interactions among age of animal, kind of diet, and level of intake. These observations, made under conditions, which, because of the long period, probably represent a more stable relationship between the diet and the body composition of the animals, suggest that the effects of drastic changes in protein intake on creatinine excretion reported by Van Niekerk et al. ('62) might be of a transient nature.

A study of the data obtained with all 65 sheep showed that 1 mg of creatinine was excreted per 24 hours for each 5.13 ± 0.43 gm of protein contained in the ingesta-free body (i.e., the mean output of creatinine per 24-hour period was 0.195 mg/gm of body protein).

Relation between body protein and urinary creatinine. A statistical analysis of the data obtained for the 65 sheep re-

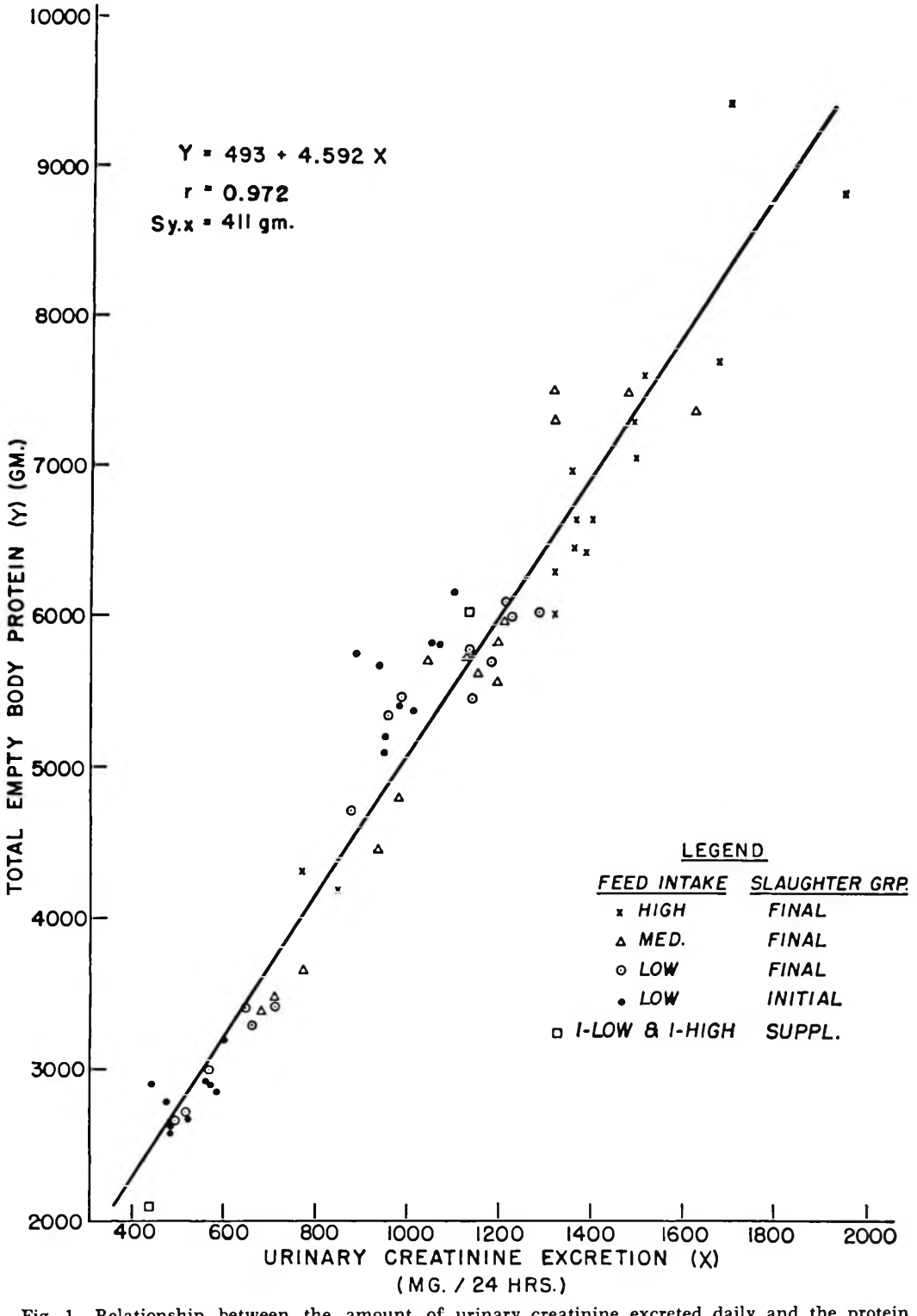

Fig. 1 Relationship between the amount of urinary creatinine excreted daily and the protein content of the ingesta-free body of sheep.

TABLE 3

Relation between urinary creatinine excretion rate and amount of protein in ingesta-free body of sheep

Relationship	Prediction equation[1]	Correlation coefficient	Standard error of estimate	Coefficient of variation
			gm	%
Linear	$Y = 493 + 4.59X$	0.972	411	7.9
Curvilinear	$Y = 3.018X + 3459 \log_{10} X - 8204$	0.974	398	7.6

[1] Y = total protein (gm) in whole, ingesta-free body, and X = urinary creatinine (mg/24 hours).

vealed that the daily excretion of urinary creatinine is highly correlated with the amount of protein (nitrogen × 6.25) in the ingesta-free body. This relationship is shown in figure 1. Equations for predicting body protein and other statistical data concerned with this relationship are summarized in table 3. The equation computed for the curvilinear relation improved significantly at the 5% level of probability the accuracy of the prediction of the linear model.

As shown in table 3, for the linear relationship, the Y-intercept value is 493 gm. It is a reasonable postulate that this value might reflect that fraction of body protein which is not directly associated with urinary creatinine. It is expected that such structural proteins as those in wool, hooves, connective tissue and the organic matrix of bones would not contribute much, if any, to creatinine production.

To what extent the results of this experiment would conform to those obtained with other species is not known. However, a comparison of the present data obtained with sheep with those obtained by Kumar et al. ('59) with rats suggests that the rate of creatinine excretion per unit of body protein is different for the two species.

Body weight as a predictor of body protein. An evaluation of indirect methods of estimating body composition requires an assessment of the degree of refinement that can be gained by an indirect method additional to the accuracy of estimating body composition from only the body weight. Such a study was made with respect to estimating the protein content of sheep from the output of urinary creatinine.

In the present study the correlation coefficient between the shrunk (after an 18- to 20-hour fast) body weight (X) and the protein content (Y) of the body is 0.969 for the total population (i.e., 65 sheep). The regression of protein content (gm) on body weight (kg) yielded the following equation: $Y = 525 + 112.4X$. The standard error of estimate was 431 gm and the coefficient of variation was 8.3%. A comparison of these data with those representing the total population shown in table 3 suggests that the error of estimating the protein content from the shrunk body weight is not much greater than that estimated from the creatinine output. However, a closer examination of the data revealed that the protein concentration of the shrunk body decreased progressively and markedly as the body weight and concentration of fat increased. Body weight was an effective predictor of the protein content of animals weighing up to 55 kg. Above this body weight the concentration of fat ranged generally from 28 to 47% with a consequent reduction in the protein concentration of the body.

An arbitrary division of the total data into one group consisting of those for sheep containing less than 28% of fat and another comprising the data for sheep containing more than 28% of fat demonstrates the inadequacy of body weight, and simultaneously the superiority of creatinine output, as a predictor of body protein in sheep containing 28 to 47% of fat. The regression of the protein contents on the body weights of the 48 sheep containing less than 28% of fat resulted in the following equation:

$Y = 132.9X - 110$; where, Y = protein content (gm) and X = body weight (kg).

The standard error of estimate was 261 gm of protein and the coefficient of variation was 5.64%. The use of this equation to predict the protein content of the 17 sheep containing from 28 to 47% of fat resulted in a mean overestimation of

12.3%. An analysis of the paired data (i.e., estimated vs. measured protein contents) for the 17 sheep revealed that the estimated values were highly significantly ($P < 0.001$) greater than the measured protein contents. Further, the correlation between the fat concentration of the body and the difference between the measured and estimated protein content expressed as a percentage of the measured protein content was 0.853.

The regression of protein content on the creatinine output in the urine of the 48 sheep containing less than 28% of fat resulted in the equation: $Y = 4.821\ X + 315$; where, Y = body protein (gm) and X = urinary creatinine (mg/day). The standard error of estimate was 387 gm of protein and the coefficient of variation was 8.37%. The use of this equation to predict the protein content of the 17 sheep containing from 28 to 47% of fat resulted in an average overestimation of 3.4%. An analysis of the paired data showed that the estimated protein content was not significantly different from the measured values at the 5% level of probability. Only an insignificant coefficient of correlation (-0.116) existed between the fat concentration and the difference between the measured and estimated protein contents expressed as a percentage of the measured protein contents. Thus, although body weight was an accurate predictor of the protein content of sheep of the kinds used in these experiments, provided they contain less than 28% of fat (and weigh less than 55 kg), the urinary creatinine output is the more accurate predictor of the protein content of fatter sheep.

A consideration of both body weight and creatinine excretion as the independent variables for the total population (65 sheep) resulted in the following multiple regression equation for predicting the protein content of the empty body:

$Y = 406 + 2.5258\ X_1 + 53.0\ X_2$; where, Y = empty-body protein (gm), X_1 = urinary creatinine (mg/day), and X_2 = shrunk body weight (kg).

For this relationship, the multiple correlation coefficient was 0.981, the standard error of estimate was 344 gm of protein, and the coefficient of variation was 6.6%. The predictive value of the multiple regression equation was found to be significantly greater ($P < 0.005$) than that of the equations for the total population in which only body weight or the creatinine output is the independent variable. A further reduction in the prediction error by considering creatinine excretion in addition to body weight is prevented by the influence of body fat on the relation between body weight and body protein.

Prediction of empty-body water and fat-free mass. Since urinary creatinine and body protein are highly correlated and since it had been shown that water and protein constitute constant fractions of the fat-free bovine body of a given age (Reid et al., '55), it was anticipated that creatinine excretion should be highly related also to the water content of the body. Also, since water and protein comprise most of the fat-free mass of the empty body, it was expected that the urinary output of creatinine would reflect the size of the lean-body mass.

In this study the amount of creatinine excreted in the urine was highly related linearly with both the water content (fig. 2) and the fat-free mass (fig. 3) of the ingesta-free body of sheep. The prediction equations, correlation coefficients, and indices of variation in these relationships are summarized in table 4. No improvement in the accuracy of prediction at the 5% level of probability was effected by fitting a curvilinear function to these data. It is noteworthy that these relationships were independent of the size or degree of fatness of the animals studied and, therefore, of the nutritional history.

These observations indicate that certain components of body composition can be estimated from the urinary creatinine excretion rate with sufficient accuracy for the latter to have great utility in certain kinds of genetic and nutritional investigations. It is expected that this index would serve particularly to refine long-term feeding experiments concerned with the utilization of protein and energy by ruminants as well as to provide an index of meat quality. In contrast with the dilution methods, the use of creatinine excretion as an index of body composition does not require the injection of reference materials which might remain in the tissues as

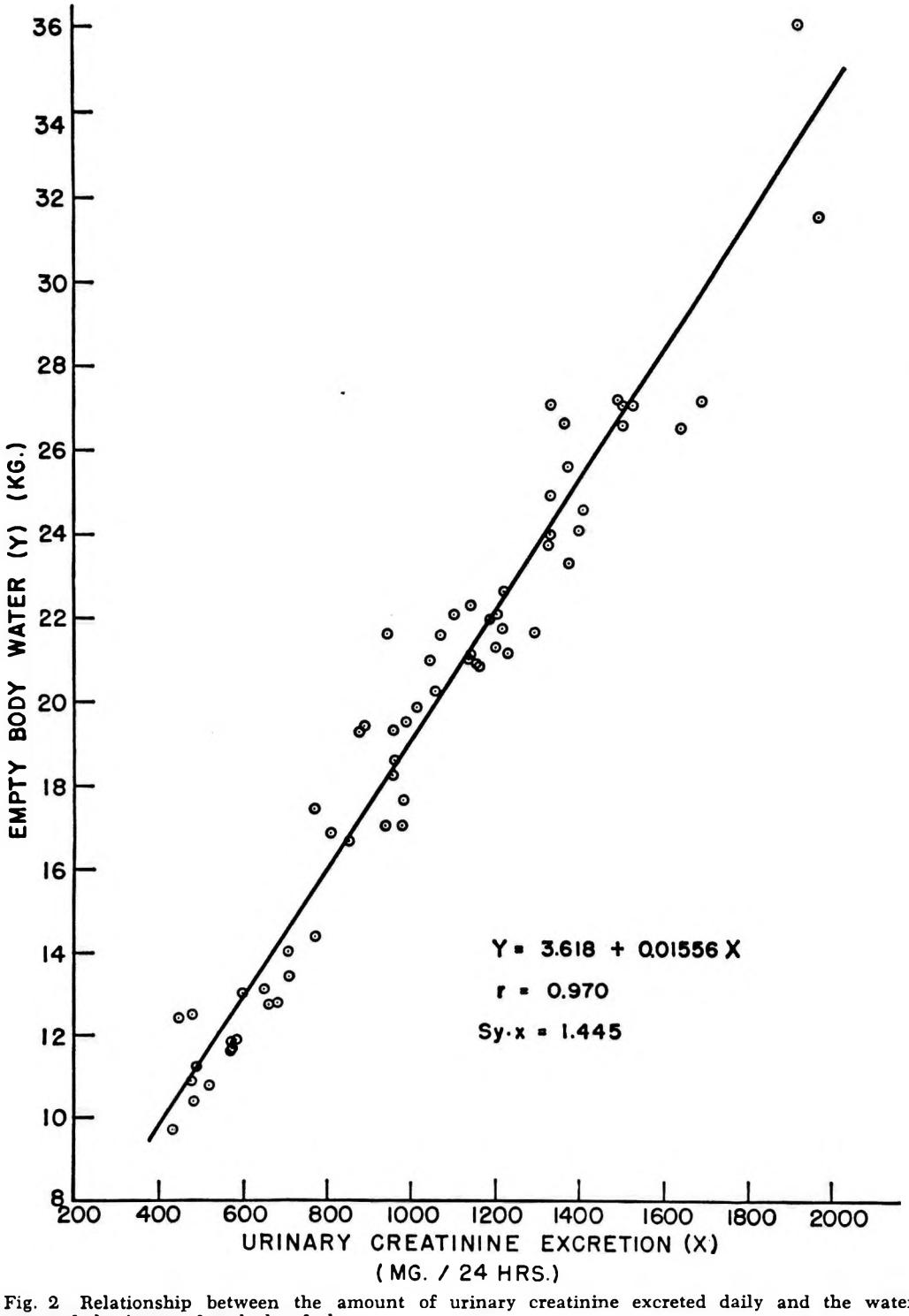

Fig. 2 Relationship between the amount of urinary creatinine excreted daily and the water content of the ingesta-free body of sheep.

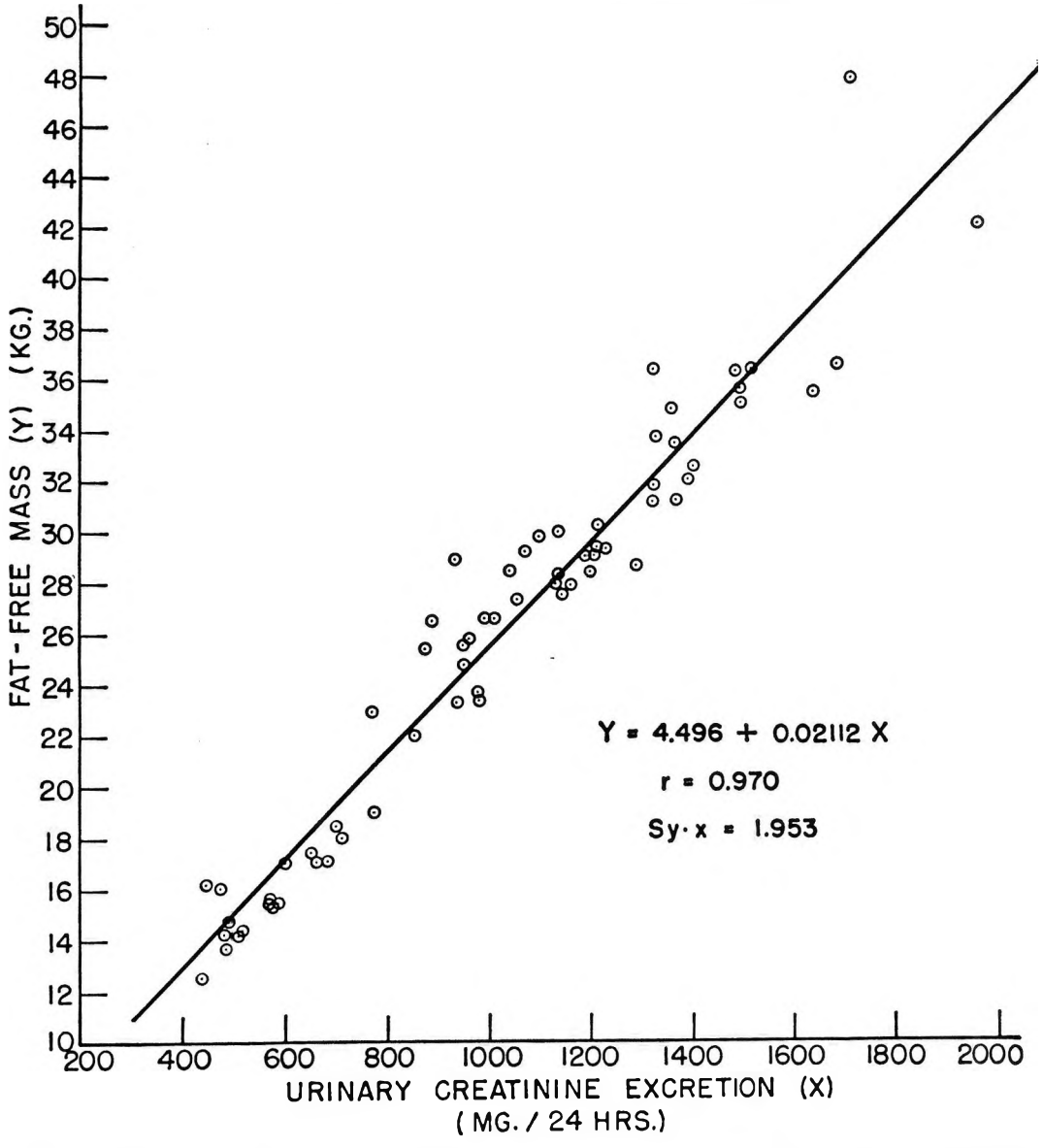

Fig. 3 Relationship between the amount of urinary creatinine excreted daily and the fat-free mass of the body of sheep.

TABLE 4

Relation between urinary creatinine excretion rate and amount of water and fat-free mass in ingesta-free body of sheep

Component predicted	Prediction equation[1]	Correlation coefficient	Standard error of estimate	Coefficient of variation
			gm	%
Water	Y = 3618 + 0.01556 X	0.970	1445	7.4
Fat-free mass	Y = 4496 + 0.02112 X	0.970	1953	7.5

[1] Y = Water (gm) or fat-free mass (gm) in whole, ingesta-free body, and X = urinary creatinine (mg/24 hours).

an undesirable residue, or the sampling and analysis of blood.

Relationship between urinary creatinine and other body constituents. There appears to be no obvious way of estimating directly and accurately the fat content of the empty body from the creatinine excretion rate. However, when the amount of digestive-tract contents is known, the fat content of the empty body can be obtained indirectly as the difference between the empty-body weight and the fat-free mass of the empty body or from the water content.

The amount of body ash was only moderately correlated ($r = 0.77$) with the creatinine output in urine and the prediction equation relating these two variables had a coefficient of variation of 19.3% of body ash.

SUMMARY

The relationships between the amount of creatinine excreted in the urine and the quantities of the major chemical components of the ingesta-free body (determined by direct analysis) were investigated in 65 sheep. The effects of age of animal, kind of diet and level of feed intake on the rate of creatinine excretion were also studied.

These studies revealed that the protein and water content and the fat-free mass of the ingesta-free body are highly correlated with the amount of creatinine excreted in the urine. Prediction equations for these relationships were derived which permit an accurate estimate of the quantity of protein, water, and the fat-free mass of the empty body of living sheep. Body weight was an accurate predictor of protein content in sheep containing less than 28% of fat and weighing less than 55 kg. As an index of protein content in sheep containing 28 to 47% of fat, creatinine output was superior to body weight. Thus, the rate of urinary creatinine excretion is an index of much promise for use in nutritional and genetic investigations with ruminants. Although no direct means of estimating body fat from the creatinine output was found, body fat may be estimated indirectly from an estimate of the fat-free mass or of the water content.

The relationships observed between urinary creatinine output and the weights of protein, water and fat-free mass in the empty body were independent of the age of sheep between 4 and 27 months, the kind of diet ingested, the level of feed intake, the size of animal, and the degree of body fatness. The animals used in these studies ranged in body weight from 17.1 to 75.8 kg and in fat content from 4.9 to 46.6%.

One milligram of creatinine was excreted per 24 hours for each 5.13 ± 0.43 gm of protein contained in the ingesta-free body.

ACKNOWLEDGMENTS

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Vitamin A and Cholesterol Absorption in the Chicken[1]

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Kinley and Krause ('59) reported that the administration of high levels of vitamin A to human beings for several months reduced hypercholesterolemia in atherosclerotic subjects but had no effect on blood cholesterol level in individuals with normal blood levels. Wood and Topliff ('61) observed that vitamin A lessens the degree of hypercholesterolemia produced in chickens by cholesterol feeding. Conversely, Green et al. ('57) showed that feeding cholesterol lowers the level of vitamin A in the livers of male rats. Subsequently Horner and Morton ('60) demonstrated that liver storage of vitamin A is affected only if vitamin A and cholesterol are given simultaneously, but not when vitamin A is administered separately and the cholesterol given later in a vitamin A-free diet.

The following experiments using chickens were designed to determine whether large doses of vitamin A prevent the circulation of high levels of cholesterol in the blood, or whether vitamin A interferes with the absorption of cholesterol.

EXPERIMENTAL

Experiment 1. Twenty-four 7-week-old cockerels of similar body weight were distributed at random into 4 groups of 6 birds each. They were fed a low-cholesterol diet[2] containing normal levels of vitamin A and carotene. The groups were subjected to the following treatments: (1) control; (2) diethylstilbestrol; (3) vitamin A; and (4) diethylstilbestrol plus vitamin A.

Diethylstilbestrol was injected subcutaneously once daily at a level of 4 mg/dose in 0.5 ml corn oil. Vitamin A was given orally by pipette twice daily (except Saturday and Sunday, when only one dose was given) at a level of 18,300 USP units per dose. The vitamin A was administered as an aqueous emulsion of a vitamin A palmitate concentrate. The emulsion was prepared with 1% of cellulose gum[3] and contained 0.05% of α-tocopheryl acetate and 0.05% of propyl gallate as antioxidants. The water in the emulsion was deaerated and saturated with nitrogen before use and the emulsion was homogenized under nitrogen. Those cockerels which were not treated with diethylstilbestrol were injected with corn oil and those not receiving vitamin A were dosed with the aqueous carrier. The control birds were given both corn oil and the aqueous carrier.

On the twelfth day of treatment blood samples were taken for determination of serum lipid and cholesterol levels (table 1). Total lipid level was measured by the turbidimetric method of Huerga et al. ('53) and cholesterol by the method of Zlatkis et al. ('53) as modified by Rosenthal et al. ('57).

TABLE 1

Effects of diethylstilbestrol and vitamin A on serum levels of total lipids and cholesterol

Treatment	Total lipid	Cholesterol
	mg/100 ml	*mg/100 ml*
Control	455 ± 42[1]	180 ± 14
Diethylstilbestrol	4290 ± 58	1085 ± 80
Vitamin A	480 ± 34	190 ± 15
Diethylstilbestrol + vitamin A	4195 ± 193	1075 ± 153

[1] Mean ± SD.

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[1] Supported by a grant from the National Research Council of Canada.

[2] Formula of diet/100 gm: (in grams) wheat screenings, 76.25; soybean oil meal (44% protein), 14.0; herring meal (73% protein), 2.0; dehydrated cereal grass (265 μg carotene/gm), 2.0; iodized salt, 0.5; distiller's dried solubles, 2.0; limestone, 1.0; bonemeal, 2.0; feeding oil (2,250 USP units vitamin A, 300 ICU vitamin D_3/gm), 0.25; and (in milligrams) manganese sulphate, 13.2; Zn bacitracin, 0.55; and riboflavin, 0.22.

[3] Type 70 C, Hercules Powder Company.

Experiment 2. Twenty-four 7-week-old pullets of similar body weight were distributed at random into 4 groups of 6 birds each. The groups were subjected to the following treatments: (1) control diet; (2) control diet plus 1% cholesterol; (3) control diet and vitamin A; and (4) control diet plus 1% cholesterol and vitamin A.

The control diet was the same as that fed in experiment 1.

The vitamin A was adminstered by pipette in an aqueous emulsion prepared as in the previous experiment. One dose of 18,300 USP units was given daily. The birds not receiving vitamin A were given the aqueous carrier.

On the basis of the cholesterol levels in blood samples taken on the eleventh day of the experiment (table 2) the birds did not appear to be absorbing any appreciable amount of cholesterol. On the thirteenth day of the test, therefore, 10% of corn oil was added to both the basal diet and to the diet with 1% of cholesterol.

Six days after the corn oil was added to the diets blood samples were analyzed for cholesterol by the method used in experiment 1. The following day the birds were killed and the portion of the intestine between the duodenum and the cecal junction was removed. The intestine was split open and the contents washed out with physiological saline. The liver and the intestine were freeze-dried, ground and extracted with petroleum ether (B.P. 30 to 60°C). The liver extracts were analyzed spectrophotometrically for vitamin A. Since the spectrophotometric readings were not corrected for extraneous absorption the values obtained can only be regarded as relative. Cholesterol was determined on the extracts of the livers and intestines according to Sperry and Webb ('50).

Experiment 3. This experiment was essentially a repetition of experiment 2 except that corn oil was included in the diets from the beginning of the test. Serum cholesterol was measured in blood drawn on the seventh day of the test. Pooled samples from each group were analyzed by the method of Sperry and Webb ('50). Two days later the birds were killed and the livers and intestines removed and analyzed for vitamin A and cholesterol as before (table 3).

RESULTS AND DISCUSSION

The serum levels of total lipid and cholesterol in the birds receiving the different treatments are shown in table 1. High levels of circulating cholesterol were induced by diethylstilbestrol without adding cholesterol to the diet. Any effect of vitamin A on serum cholesterol level would therefore have been independent of exogenous cholesterol absorbed from the intestine. Injection with diethylstilbestrol resulted in the expected increases in both total lipid and cholesterol, whether or not the birds were given large amounts of vitamin A. In birds fed a low-cholesterol diet, vitamin A apparently did not mediate against the circulation of high levels of cholesterol. The effect of a high level of vitamin A upon the absorption of exogenous cholesterol was accordingly studied in the next experiments.

In experiment 1 hypercholesterolemia was achieved with a low-cholesterol diet. In experiments 2 and 3, however, serum

TABLE 2

Cholesterol levels in the serum, intestinal wall and liver of chicks in experiment 2

Treatment	Cholesterol level			
	Serum[1]	Serum[2]	Intestinal wall	Liver
	mg/100 ml	*mg/100 ml*	*mg/100 gm*	*mg/100 gm*
Control	191 ± 23[3]	213 ± 9	338 ± 20	289 ± 19
1% Cholesterol	222 ± 56	515 ± 208	718[4] ± 187	2079 ± 1051
Vitamin A	182 ± 17	194 ± 22	322 ± 15	232 ± 108
1% Cholesterol + vitamin A	241 ± 90	396 ± 126	541[4] ± 121	1715 ± 1313

[1] Before the addition of corn oil to the diets.
[2] After the addition of corn oil to the diets.
[3] Mean + SD.
[4] The difference between these treatments is significant ($P < 0.05$) according to analysis of variance with the corresponding groups in experiment 3.

TABLE 3

Cholesterol levels in the serum, intestinal wall and liver of chicks in experiment 3

Treatment	Cholesterol level[1]		
	Serum	Intestinal wall	Liver
	mg/100 ml	*mg/100 gm*	*mg/100 gm*
Control	111	333 ±15	324 ±9
1% Cholesterol	292	652[2] ±66	2429[3] ±746
Vitamin A	122	361 ±30	324 ±13
1% Cholesterol + vitamin A	241	554[2] ±87	1433[3] ±753

[1] Mean ± SD.
[2] The difference between these treatments is significant($P < 0.05$) according to analysis of variance with the corresponding groups in experiment 2.
[3] Difference significant ($P < 0.05$).

cholesterol was increased by feeding 1% of cholesterol in the diet. The results are presented in tables 2 and 3.

Prior to the addition of corn oil to the diets in experiment 2 the birds evidently absorbed little cholesterol. Although a small amount of cholesterol may be absorbed in animals fed a fat-free diet, it has been shown repeatedly that dietary cholesterol does not effectively promote hypercholesterolemia unless the diet likewise contains a high level of fat.

Following the addition of corn oil to the diets, the cholesterol levels in the serum of the birds receiving cholesterol increased markedly. The increase in cholesterol level was greater, however, in the birds that were not given supplementary vitamin A. The amount of cholesterol present in the intestinal wall and in the liver was also greater in the birds not receiving the high level of vitamin A. The absorptive capacity of the intestinal wall had evidently been reduced by the vitamin A and cholesterol absorption was consequently retarded.

In experiment 3 serum cholesterol was measured by the method of Sperry and Webb ('50) because vitamin A is reported to interfere in the determination using ferric chloride (Kinley and Krause, '58). The results of experiment 3 (table 3) show that, as in the previous experiment, large doses of vitamin A moderated the increase in serum cholesterol resulting from feeding 1% of cholesterol. The amount of cholesterol in the intestinal wall and in the liver was also lower when the cholesterol-fed birds were given supplementary vitamin A.

Statistical analysis of the cholesterol levels in the livers of individual birds showed that the difference between the birds not receiving additional vitamin A and those given large doses of vitamin A were statistically significant only in experiment 3. The relationship between liver cholesterol levels and the levels in the intestinal wall of the birds in the two experiments was significant and is shown graphically in figure 1. In these experiments, therefore, the amount of cholesterol present in the intestinal wall provides a measure of the amount of cholesterol absorbed by the chicken. Without the observation that the level of cholesterol in the liver is positively related to the level in the intestinal wall, differences in the amount found in the intestinal wall could not be assumed to be indicative of differences in absorption across the wall.

The relative levels of vitamin A present in the livers of the birds fed the diets with and without cholesterol are shown in table 4, and indicate that the interference

TABLE 4

Liver storage of vitamin A

Treatment	Experiment 2	Experiment 3
	USP units/ gm tissue	*USP units/ gm tissue*
Control	404[1] ±55[2]	420[3] ±69
1% Cholesterol	291[1] ±34	315[3] ±69
Vitamin A	5530 ±674	1893[4] ±487
1% Cholesterol + vitamin A	5447 ±771	1217[4] ±218

[1] Difference significant ($P < 0.01$).
[2] Mean ± SD.
[3] Difference significant ($P = 0.05$).
[4] Difference significant ($P < 0.05$).

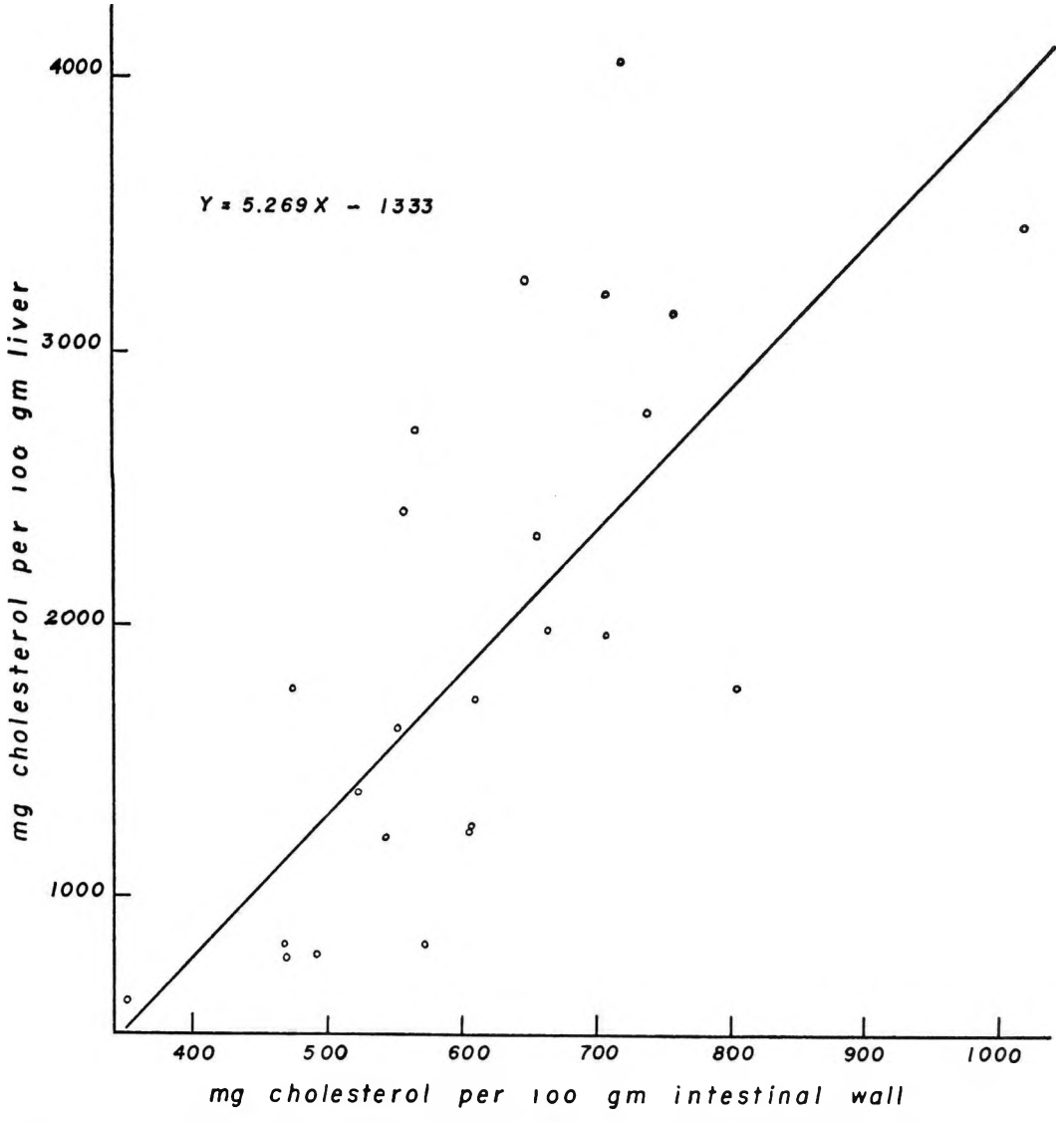

Fig. 1 Regression of the level of cholesterol in the liver on the level of cholesterol in the intestinal wall.

between vitamin A and cholesterol is mutual. The lower vitamin A content of the livers in the birds fed 1% of cholesterol is in agreement with the data on rats (Green et al., '57; Horner and Morton, '60). In experiment 2 the relatively high levels of liver vitamin A in the birds given supplementary vitamin A by pipette were due to the fact that in this experiment the chicks were on test for 20 days, whereas experiment 3 was of only 8 days' duration. Furthermore, during the first 13 days of experiment 2 the birds receiving cholesterol and vitamin A were, on the basis of their serum cholesterol levels, not absorbing appreciable cholesterol. The similarity in the vitamin A levels in the livers of the birds fed the high level of vitamin A, whether or not cholesterol was fed, is accordingly in agreement with the observation of Horner and Morton ('60) that vitamin A stores formed before cholesterol was fed to rats were not affected by the cholesterol. However, in the birds of ex-

periment 2 that received a normal level of vitamin A in the diet, 1% of cholesterol did significantly reduce liver storage of vitamin A. It seems probable, therefore, that the high level of vitamin A already stored before the birds were given cholesterol in a readily absorbable form masked any effect of cholesterol in the latter part of the experiment after the feeding of corn oil.

SUMMARY

The administration of large amounts of vitamin A to 6- to 7-week-old chickens reduced the increase in serum cholesterol level resulting from the inclusion of 1% of cholesterol in the diet. Vitamin A did not, however, moderate the hyperlipemia and hypercholesterolemia produced in birds fed a low cholesterol diet and treated with diethylstilbestrol. Large doses of vitamin A reduced the level of cholesterol in the intestinal wall and in the liver of birds fed 1% of cholesterol. Conversely, birds receiving large amounts of vitamin A and 1% of cholesterol in the diet did not store as much vitamin A in the liver as birds receiving the same amount of vitamin A in conjunction with a diet low in cholesterol. It is concluded that there is mutual interference between vitamin A and cholesterol during the course of absorption across the intestinal wall.

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Studies in Infantile Malnutrition

I. NATURE OF THE PROBLEM IN PERU[1]

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Dietary surveys and food balance studies yield information of use in the evaluation of the diet of entire populations but do not tell us much about the nutritional status of small groups within those populations and particularly of the different age categories. Direct observation, by physical examination and anthropometric measurement of large numbers of representative individuals, should single out the deficient groups within a population, but this is a task of great proportions, particularly if one hopes to examine a significant number of the infants and pre-school children. On the other hand, careful study of typical cases of malnutrition can give us valuable information about its causes and characteristics, thus reinforcing information obtained by other, indirect means.

We have completed detailed observations on 75 severely malnourished infants in Peru and believe that this information will prove useful in characterizing malnutrition in this country and in planning for its treatment and prevention. Forty-two of these infants were seen at the Hospital del Niño in Lima and all presented with severe malnutrition, diarrhea and dehydration. Forty of these were cases of kwashiorkor who were admitted because of serious derangements of electrolyte and water metabolism and the other two were marasmic infants admitted for similar reasons to the hospital. This should not be construed as representative of the relative incidence of these two conditions in Peru, as infants with marasmus far outnumber those with kwashiorkor; these, however, tend to become more dramatically ill and are more readily admitted to the limited number of beds available. The remaining 33 cases, 11 of kwashiorkor and 22 of marasmus, were observed in a metabolic ward at the British American Hospital, where they were admitted during the course of studies on diarrhea and malnutrition. They were referred from the Out-patient Department of the Hospital del Niño or directly from dispensaries in the slums surrounding Lima.

RESULTS

Clinical characteristics. The cases classified as kwashiorkor all had edema, hypoalbuminemia and typical skin and hair changes. Those listed as marasmus all were free of apparent edema, nearly always had normal serum albumin and had weight deficits of at least 40% for their age. One infant, age 23 months, and weighing 6.2 kg, had sparse reddish hair which fell out easily and also had a fading "peeling paint" dermatosis. She had no edema and had a normal serum albumin; 11 months before admission she had had measles and we suspect that shortly after this illness she may have been edematous, thus representing in effect a partly recovered case of kwashiorkor. One other infant, age 30 months, and weighing 5.7 kg on admission, had a long history of vomiting during the course of which he had spent 8 months in a hospital with a presumptive diagnosis of encephalitis. Since these two cases were not at all typical of the remaining group with marasmus, in tables 1 to 4 this group has been considered with and without these two cases and in figure 1 the same two cases have been excluded.

Family history. Although accurate information is available on only a limited number of the families, their economic

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situation is uniformly bad: all are slum dwellers, with the entire family living and sleeping in a single room or two, with two to four persons per bed. They derived their livelihood from the meager sum contributed by the mother's legitimate or common-law husband, or more likely than not, from her own work, and usually they received some food from the parish priest or other charitable organizations. The daily per capita income, when it could be ascertained, was seldom enough to buy one liter of raw milk, least of all a safer preparation. When reliable information was available, the majority of infants were reportedly illegitimate, with an apparently higher frequency in the group with kwashiorkor. The number of children per family (table 1) was greater for the group with marasmus (5.0 vs. 3.9) and the number of very large families (6 or more children) was notably higher in this same group. It is also striking that there were no families with a single child and that only one of the cases of kwashiorkor was the first born in his family. The relatively late position in order of birth of most of our cases suggests a deteriorating family economic situation. Extreme poverty, accentuated by a large number of children to feed, plays a leading role in the malnutrition of these infants. The higher mortality rate among the siblings of those with marasmus suggests that they came from a poorer environment. Although we have not examined the living siblings of our patients, we have seen enough of them to describe them as short of stature but relatively well nourished in most cases.

Physical characteristics. Table 2 summarizes the anthropometric measurements of the entire group on admission as well as their deviation from normal. There are accurate figures on the heights and weights of Peruvian children of the same racial background (Indian or Mestizo) during the first two years of life and under nearly ideal living conditions (Lozano, '59). During the first 9 months of life these measurements fall between the 25th and the 50th percentiles for U. S. children, but during the second year the average is near the 10th percentile, suggesting a racial difference, poorer nutrition, or perhaps poor sampling. Since there is no standard of similar accuracy available for head circumference of Peruvian children,

TABLE 1

Family characteristics of 51 cases of kwashiorkor and 24 cases of marasmus

Type of malnutrition	Kwashiorkor	Marasmus (total)	Marasmus (typical)[1]
Total no. of cases	51	24	22
Birth status: legitimate	2(4%)	9(37%)	7(32%)
illegitimate	9(18%)	9(37%)	9(41%)
not known	40(78%)	6(25%)	6(27%)
Sex of patient: male	25(49%)	11(46%)	10(45%)
female	26(51%)	13(54%)	12(55%)
Avg no. children in family	3.9	5.0	5.0
Range	2–10	2–12	2–12
No. of siblings:[2] living	91(93%)	81(84%)	76(86%)
dead	7(7%)	15(16%)	12(14%)
total	98	96	88
Patient's order of birth:[2]			
first born	1(3%)	—	—
second	5(15%)	1(4%)	1(5%)
third	15(44%)	7(29%)	6(27%)
fourth	6(18%)	6(25%)	6(27%)
fifth	3(9%)	—	—
sixth or later	4(12%)	10(42%)	9(41%)
last born	28(82%)	23(96%)	22(100%)

[1] Two atypical cases of marasmus (see text) are not included.
[2] Data available on only 34 of 51 patients with kwashiorkor.

TABLE 2

Body measurements on admission of 51 cases of kwashiorkor and 24 cases of marasmus

	Kwashiorkor	Marasmus (total)	Marasmus (typical)[1]
Body wt			
Avg after diuresis	7.2 kg	4.5 kg	4.3 kg
Range	5.0–10.0	2.9–6.6	2.9–6.6
Avg age	22.6 months	12.0 months	10.6 months
No. cases (range)	51(10–58)	24(6–30)	22(6–20)
Avg "weight age"	6 months	1 month	1 month
Range	2–13	0–4	0–4
% of admission age	27%	8%	9%
Body length			
Avg	74.1 cm	63.3 cm	62.3 cm
Range	66–86.5	54–75.2	54–70.5
Avg age	22.5 months	12.0 months	10.6 months
No. cases (range)	49(10–58)	24(6–30)	22(6–20)
Avg "height age"	12 months	5 months	4 months
Range	6–23	1–13	1–9
% of admission age	53%	42%	38%
Head circumference			
Avg	44.4 cm	40.0 cm	39.7 cm
Range	40.5–48	37–44	37–44
Avg age	22.4 months	11.9 months	10.6 months
No. cases (range)	44(10–58)	22(6–30)	20(6–20)
Avg "head age"	8 months	3 months	2.5 months
Range	3–24	1–6	1–6
% of admission age	35%	25%	23%
Teeth			
Avg no. erupted	13.5	4.75	3.6
Range	2–20	0–18	0–14
Avg age	21.9 months	12.0 months	10.6 months
No. cases (range)	44(10–58)	24(6–30)	22(6–20)
Avg "dental age"	18 months	9 months	9 months
% of admission age	82%	75%	85%
Bone maturation			
Avg age	20.9 months	12.0 months	10.6 months
No. cases (range)	11(11–29)	22(6–30)	20(6–20)
Avg "bone age"	13.0 months	4.75 months	4.25 months
Range	3–26	2–12	2–9
% of admission age	62%	40%	40%

[1] Two atypical cases of marasmus (see text) have been excluded.

it was decided to use a well-known U. S. standard (Anthropometric Chart, The Children's Medical Center, Boston) as a point of reference for all three body measurements. Since all our patients were so severely retarded in their development, their measurements all fall within a range which corresponds to ages in which there is very little difference between U. S. and Peruvian standards. Consequently the average for each dimension in each group has been expressed as the age at the 50th percentile of U. S. standards to which it would correspond. If the Peruvian standard for weight had been used, the "weight age" would have been identical in each category. In the case of height, the difference between the U. S. and the Peruvian standard would never have exceeded one month. For further comparison, in table 2 the apparent age has also been expressed as a percentage of the chronologic age. In the case of weight, the lowest weight reached during the hospitalization has been used as the point of reference. In most of the cases of kwashiorkor this is significantly lower than the admission weight, which invariably included a large amount of edema fluid and could not be taken as a true index of the body mass. For dental age the number of erupted teeth has been translated

into the age range with this number of teeth usually present.

The infants with marasmus were almost exactly one year younger than those with kwashiorkor and showed significantly more retardation in all measurements except dental age. In neither group was there a significant deviation from normal in this respect: the eruption of teeth in humans, as in animals (McCance and Widdowson, '62) appears to proceed independently of the state of nutrition. Retardations in height and bone age were almost exactly parallel in both groups, whereas weight age was considerably more retarded than either, being quite extreme in the marasmic group.

The very significant retardation in head circumference may have important implications; it is apparently more notable than that in height and bone age, although it is possible that at least in part this may be due to racial differences. The partial data that exist on head circumference of "normal" children of the same racial group indicate very little difference from the U. S. standard we have used, suggesting that this retardation is indeed real. Nearly all of these infants showed significant mental retardation and our subsequent observations have shown very little if any "catching up" in this respect; this has been particularly absent in the marasmic infants with the greatest deficits in height and head size at the time of admission. It has been shown (Lat et al., '60) that animals that are underfed early in life always remain undersize and show definite limitations in their learning skill. Undoubtedly the poor environment and the neglect suffered by these infants must play a part in their slow mental development; but we are under the impression that severe malnutrition at such early ages must adversely affect the development of the brain as in the case of congenital cretinism.

Feeding history. The most striking contrast between these two groups of malnourished infants is in their dietary history as illustrated in table 3 and figure 1. Although in the group with kwashiorkor

TABLE 3

Feeding history of 51 cases of kwashiorkor and 24 cases of marasmus

	Kwashiorkor	Marasmus (total)	Marasmus (typical)[1]
Total no. of cases	51	24	22
Exclusively breast fed[2] for one month or more	46(90%)	5(21%)	3(14%)
Avg duration of exclusive breast feeding periods	7.8 months	3.8 months	2.0 months
Range in months	1–24	1–11	1–3
Mixed feeding[3]	2(4%)	10(42%)	10(45%)
Exclusive and/or mixed	48(94%)	15(62%)	13(59%)
Avg duration of periods of breast feeding	9.6 months	3.7 months	2.8 months
Range in months	1–30	1–18	1–8
Artificially fed[4]	3(6%)	9(37%)	9(41%)
Avg duration of exclusive breast feeding for total group	7.0 months	0.8 month	0.25 month
Avg duration of any breast feeding for total group	9.0 months	2.3 months	1.5 months

[1] Two atypical cases of marasmus (see text) have been excluded.
[2] The term "exclusive" breast feeding indicates that no other milk was regularly given during these periods.
[3] The term "mixed" breast feeding indicates that some form of cow's milk was regularly used to supplement the mother's milk during these periods.
[4] The term "artificial" feeding indicates that mother's milk was not given beyond the age of one month.

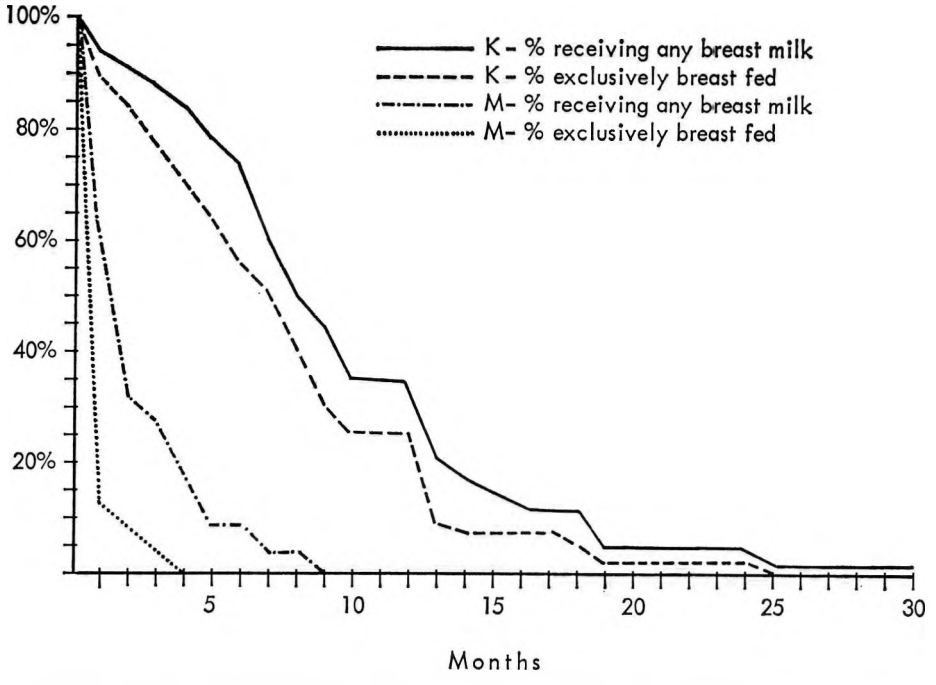

Fig. 1 Incidence of breast feeding (% of all cases) from birth to 30 months of age in 51 cases of kwashiorkor (K) and 22 cases of marasmus (M). Many of the "exclusively" breast fed infants received foods other than milk, usually broths.

there appears to have been a high rate of prolonged breast feeding, the incidence of successful maternal nursing in the cases of marasmus was practically nil. In both groups, when the breast failed or was no longer offered, cow's milk preparations of very dubious value were generally used for variable intervals, but in most cases the mother eventually attempted to replace the breast with watery soups and teas, cornstarch and other hopelessly inadequate foods, both as to protein content and as to caloric value. The methods described for preparing "formulas" were extremely varied and seldom verifiable: it was common for a mother to state that she used equal parts of evaporated cow's milk and water for her infant and in the next breath admit that a 13-ounce can of evaporated milk lasted the family for three days. In most cases of both groups it is possible to show a relation between the duration of exclusive breast feeding and the size achieved by the infant. One of the most serious situations encountered is that of the working mother who leaves a small infant locked in the home under the care of the oldest sibling, very often a girl of 7 or 8 years of age. The mother may offer the breast or a substitute early in the morning and again in the evening on returning from work, and during the day the infant receives little or no food. The fact that most of our cases of severe malnutrition occur in the third or fourth born infant suggests that only when her oldest child reaches the "responsible" age of 7 or 8 does the mother feel confident enough to leave her or him in charge of the house and go out to seek employment. Our observations appear to confirm this supposition.

Medical history. In this group pertussis does not appear to have played a significant role: this may be a false impression as in this area, as in all of Peru, pertussis occurs in large epidemics and reputedly accounts for many infant deaths. It is logical to assume that it must have an adverse effect on an already inadequate nutritional state, if only because of the frequent vomiting it causes. The incidence of measles, on the other hand, looms very large in the past medical his-

tory of the cases of kwashiorkor. In no less than 13 cases it occurred within the two months immediately preceding admission and in all of these cases the mother dated the onset of the child's deterioration to this illness. Measles probably produces a prolonged period of negative nitrogen balance even in the face of an adequate intake and when to this is added the tendency, both among mothers and among physicians in Peru and elsewhere, to starve children with measles and other severe illnesses, it is easy to see how it could precipitate a serious decompensation of protein metabolism in the form of kwashiorkor (Scrimshaw, '61).

Frequent episodes of diarrheal disease were very common in the past medical history of both groups but prolonged severe diarrhea of more than two weeks' duration prior to admission was definitely more common in the cases of kwashiorkor. Here it is more difficult to separate cause and effect. In the vast majority of the marasmic infants admitted with chronic diarrhea we have not been able to demonstrate any acute water deficit and the diarrhea has responded well to milk feeding, suggesting that it is only a symptom of chronic starvation and not its cause. The unfortunate habit of treating this common type of diarrhea with further starvation leads to disastrous results. In the cases of kwashiorkor this iatrogenic starvation often had precipitated the final episode of edema. Many of our cases of kwashiorkor, however, did present with acute, severe diarrhea and the grave disturbances of water and electrolyte metabolism which produce such a high initial mortality in these cases.

DISCUSSION

The foregoing information allows us to draw some tentative conclusions about the nature and causes of malnutrition in Peru as well as to make some inferences about its effect on the physical and mental well-being of its population. The great majority of cases came to our attention after six months and before two years of age. Extreme poverty and unavailability of food are of great importance, the problem being most critical in the weaned infant, whether two months or two years of age. Cow's milk production is limited and, when available, this product is beyond the economic reach of the vast majority of the population. Mothers are extremely reluctant to feed children in the first two years of life the foods that they themselves eat and consequently rely on hopelessly inadequate paps, soups and teas. Proper substitutes for breast milk are either unknown or unavailable to them. Canned fish is probably the most inexpensive animal protein available to these people, but even if they did use it, they would be hard put to meet the caloric requirements of their infants and small children. No common food can match milk in its ease of administration, continued acceptability, protein content and caloric value. When it is not available, particularly in the first year of life, the results are disastrous. We have only to consider the difficulties and expense involved in feeding a weaned infant who is sensitive to cow's milk proteins. Peruvians of all social levels are extremely reluctant to feed eggs to their children of any age.

Although there were slight differences in the characteristics of the families of infants with marasmus and those with kwashiorkor, these did not appear very significant and at least partly can be explained by the difference in average age between the two groups. In all body measurements, however, the degree of retardation in the group with marasmus was definitely more pronounced. Follow-up studies now in progress reveal that under nearly ideal conditions a good deal of the deficit in some of the measurements can be made up, but that the more marked the degree of retardation, the less likely it is to be made up. We are under the impression that the most retarded of these patients are doomed to be significantly dwarfed as adults, both physically and mentally. It is all too frequent in Peru to encounter adults who are 135-cm tall or less and whose mental age does not exceed 10 or 12 years. Inquiry as to their origin always reveals that they came from very poor environments where malnutrition was and is rampant. Studies under progress, of seemingly healthy populations in one of the richer coastal valleys show unexpectedly low averages for height and

weight in the adult population as well as the children, indicating a marked degree of undernutrition, probably most notable in early infancy and childhood.

The most striking difference between these two groups was in the incidence and duration of exclusive or even partial breast feeding. The group with marasmus can quite fairly be said to represent total failure of breast feeding, which in this environment is practically equivalent to a sentence of death from starvation or to survival as a physically and mentally diminished individual. Infants with similar racial backgrounds, weaned at an early age but living under sanitary conditions and fed artificially with good quality milk products, grow during their first year of life at a rate not unlike that of infants in the United States (Lozano, '59). The group with kwashiorkor had usually been breast fed with some success and had grown well during this time. Subsequently, nutrition had obviously been inadequate in protein, calories and other nutrients, determining a cessation of growth and a laying of the ground for acute protein deficiency.

In the medical histories of these infants we again note some striking differences. Most of the cases of marasmus had not had a particularly severe insult to their nutrition: they had gone along for months having mild chronic diarrhea, in most cases the result and not the cause of their starvation. In the last few days just prior to admission a more severe episode of diarrhea had usually placed them in a critical state and precipitated their admission to a hospital. In the cases of kwashiorkor two facts stand out: fully 25% of all cases had had measles within the two months prior to admission. In all these cases, either through maternal decision or on medical advice, food was cut off during the episode of measles, stools became more frequent and refeeding was delayed; quite soon mild to severe generalized edema developed. In 68% of cases, including some of the ones with recent measles, more severe diarrhea had persisted for at least 15 days prior to admission and had finally been complicated with generalized edema.

Recently it has become customary to refer to marasmus and kwashiorkor as two forms or stages of protein or protein-calorie malnutrition, and in our series there were many characteristics common to both, most of the cases of kwashiorkor fitting the description of what has been called marasmic kwashiorkor. For practical reasons we are reluctant to accept this unification. We would define marasmus as severe caloric undernutrition; no matter how high the intake of protein could have been made, if the total intake of food had remained as low, these infants would probably have reached the same state. In most of our cases growth had barely started when it came to a halt: in order to survive, their organisms made drastic readjustments, cutting out almost all activity, reducing their basal metabolic rate (Montgomery, '62) and prolonging the life of their body proteins, as has been demonstrated in animals maintained on grossly inadequate intakes (Vaughan et al., '62). When re-fed, whether they receive 50, 75 or 100 Cal./kg of body weight a day, their weight remains almost constant, regardless of the protein intake, while their basal metabolic rate is gradually increasing (Montgomery, '62). Only when their caloric intake is markedly increased do they begin to grow. It is undoubtedly possible for an infant who has been well nourished for some time and then suffers a gradual but drastic reduction in his caloric intake to become "marasmic" and this does happen in Peru. It is more common, it seems to us, for the infant who has been well nourished during most of his first year of life, to suffer a reduction in the quantity and quality of his protein intake and then pass through an episode of increased catabolism, finally developing kwashiorkor. In a series of populations now under study we have thus far found a dropping off of weight and failure to grow in height between the ages of 12 and 18 months.

The typical infant with kwashiorkor, or much more commonly marasmic kwashiorkor, usually had had a significant period of good nutrition and growth while being breast fed; this may have been followed by a period of relative starvation and then a severe infection with a prolonged period of increased catabolism and negative nitrogen balance. It is probable that the turnover rate of their proteins is much

TABLE 4

Past medical history of 51 cases of kwashiorkor and 24 cases of marasmus

Type of malnutrition	Kwashiorkor	Marasmus (total)	Marasmus (typical)[1]
Total no. of cases	51	24	22
Avg admission age (months)	22.6	12.0	10.6
Pertussis, typical history of	9(18%)	1( )	0( )
Avg age at time (months)	9.3	12.0	—
Avg months before admission	11.3	11.0	—
Measles, typical history of	29(57%)	4(17%)	3(14%)
Avg age at time (months)	18.0	7.8	6.3
Range (months)	6–42	3–12	3–12
Time before admission:			
0–1 month : no. cases	5	—	—
2 months: no. cases	8	—	—
3–6 months: no. cases	5	—	—
7–12 months: no. cases	4	4	3
over 12 months: no. cases	3	—	—
unknown: no. cases	4	—	—
Diarrhea, history of			
Frequent episodes	31(61%)	18(75%)	17(77%)
Only 1 or 2 episodes	3(6%)	1(4%)	1(5%)
None	3(6%)	5(21%)	4(18%)
Inadequate history	14(27%)	—	—
Duration of last episode prior to admission:			
none	4(8%)	4(17%)	3(14%)
1–3 days	2(4%)	2(8%)	2(9%)
4–7 days	4(8%)	8(33%)	8(36%)
8–15 days	6(12%)	6(25%)	5(23%)
16–30 days	13(25%)	2(8%)	2(9%)
over 30 days	22(43%)	2(8%)	2(9%)

[1] Two atypical cases of marasmus (see text) have been excluded.

greater than that of the marasmic infants who have adjusted to very low intakes and metabolic rates and that a severe insult such as measles provokes an acute decompensation of protein metabolism, particularly if there has been a preceding period of depletion.

Although there is always danger in oversimplification, we feel that there are important implications in the treatment and prevention of severe malnutrition to be derived from maintaining this distinction and that the failure to appreciate the very serious caloric deficit of marasmic infants leads to a waste of expensive and valuable protein and to continued failure in their treatment. The insistence of many experienced pediatricians on human breast milk as the only treatment for severe marasmus is probably based on the high calorie-to-protein ratio which we, as well as other authors (Montgomery, '62) have found ideal for their treatment. More important still, since marasmus as defined above is extremely prevalent and has a grave effect on the future growth and mental development of its survivors, we feel that its prevention deserves first consideration in any nutritional program for Peru and other nations with a similar problem. Severe malnutrition at such an early age and of such frequency requires the development of substitutes for milk until the economic development of these countries makes this product available to the mass of the people in a safe form. Although some of the protein-rich foods now being developed will undoubtedly help raise the nutritional standards of these populations, they will be of little help to the young weanling unless they can be put into a form which their mothers can and will use in their feeding. We feel they should be presented as a complete infant food with a high caloric value and not just as a protein-rich supplement. Even so,

marasmus will continue to be a problem in the case of children of working mothers. This is a social problem which can be solved only by providing adequate day nurseries for these infants and children.

SUMMARY

The backgrounds and physical characteristics of 75 severely malnourished infants have been analyzed. Those presenting as marasmus had not received breast milk for any significant length of time, were virtually starved and were more severely retarded in their physical parameters than those with kwashiorkor who usually had been adequately nursed by their mothers, but then were depleted for a variable length of time until a severe infection, commonly measles or diarrhea, precipitated the clinical signs of protein deficiency. The implications of these differences in the treatment and prevention of malnutrition are discussed.

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Composition of Dietary Fat and the Accumulation of Liver Lipid in the Choline-deficient Rat[1]

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As early as 1936 Channon and Wilkinson ('36) demonstrated that the kind of fat included in a choline-deficient diet for rats had a pronounced effect upon the amount of fat deposited in the liver. They (Channon et al., '42) concluded that fat deposition was proportional to the amounts of the saturated fatty acids supplied by the dietary fat. Stetten et al. ('45) compared the effects of the ethyl esters of the saturated fatty acids in the diets of choline-deficient rats. The chain length was shown to be important and ethyl myristate produced livers with more fat than either longer or shorter chain acids. Ethyl laurate was also found to be uniquely toxic and produced a rapidly fatal myocardial lesion at the high level fed. More recently, Benton et al. ('56) found the fraction of butter fat that contained most of the saturated fatty acids to be more lipogenic than the unsaturated fraction. On the other hand, Best et al. ('58) fed a number of different fats to choline-deficient rats and concluded that wide differences in the degree of unsaturation produced only minor differences in the degree of fatty liver produced.

In this paper we are reporting the results obtained with 13 different fats or mixtures of fats, each included in a choline-deficient diet at three levels. In conformity with most of the previous work reported, the fat content of the livers of the animals varied greatly. The degree of unsaturation of the dietary fat appears to be primarily responsible for the differences observed.

EXPERIMENTAL

Thirty-nine groups of weanling male rats (Charles River CD strain) were used. Each group of 4 animals was housed in a single cage and fed the experimental diet ad libitum for two weeks, at which time they were killed and the livers removed for examination and analysis. The diet containing the highest level of dietary fat, approximately 40% of the total calories, had the following composition: fat, 20; purified casein,[2] 12; salt mixture (Hegsted et al., '41), 4; halibut liver oil,[3] 0.1; vitamin mixture, 0.5; and glucose, 57.8. No choline was added. When smaller amounts of fat were included in the diets to provide approximately 20 and 10% of the dietary calories rather than the 40% provided by this diet, isocaloric amounts of glucose were added to replace the fat removed. One-third of the animals, 13 groups, received each level of dietary fat.

TABLE 1
Degree of saturation of the fats used

	Saturated	Mono-unsaturated	Poly-unsaturated
	%	%	%
Olive oil	15.2	79.4	6.4
Safflower oil	10.1	13.5	76.4
Coconut oil	92.7	5.9	1.4

The fat in the diets was supplied as coconut oil, olive oil, safflower oil, or mixtures of these. The composition of each of the three oils as determined by gas liquid chromatography is shown in table 1.

When the animals were killed, a portion of liver, the kidney, heart and stomach were fixed in neutral buffered 10% formalin and sections were stained with

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[2] General Biochemicals, Chagrin Falls, Ohio.

[3] Haliver Oil, Abbott Laboratories, North Chicago, Illinois

TABLE 2

Effect of dietary fats on liver lipids of choline-deficient rats

Groups	Proportion of dietary fat derived from			Relative[2] unsaturation	Liver lipid, % wet weight		
	Safflower oil	Olive oil	Coconut oil		10%[1] Dietary fat	20%[1] Dietary fat	40%[1] Dietary fat
1, 2, 3	all			166.3	14.2	9.4	8.2
4, 5, 6		all		90.2	18.1	20.6	18.2
7, 8, 9			all	8.7	22.3	21.9	28.1
10, 11, 12	2/3	1/3		139.5	12.4	7.6	12.0
13, 14, 15		1/3	2/3	35.6	19.8	28.4	28.2
16, 17, 18	1/3	2/3		114.5	8.1	14.2	15.9
19, 20, 21	1/3		2/3	60.7	16.8	19.3	20.3
22, 23, 24		2/3	1/3	62.5	20.4	14.4	21.8
25, 26, 27	2/3		1/3	112.6	15.4	17.4	11.8
28, 29, 30	1/3	1/3	1/3	87.6	22.5	17.6	16.6
31, 32, 33	1/4	1/2	1/4	88.8	14.9	14.7	20.7
34, 35, 36	1/2	1/4	1/4	107.9	19.8	19.1	17.4
37, 38, 39	1/4	1/4	1/2	68.7	12.2	21.2	20.6
				Mean liver lipid	16.7	17.4	18.4

[1] Expressed as per cent of dietary calories.
[2] Per cent oleic acid + (2 × per cent linoleic acid).

hematoxylin and eosin. Some liver sections were stained with Sudan IV. The degree of fatty metamorphosis of the liver was evaluated on a zero to 4 plus basis. The remainder of the liver was used for the fat determination. Each liver was homogenized in approximately 20 volumes of a 2:1 chloroform-methanol mixture. After standing several hours at room temperature, the homogenate was centrifuged and the supernatant filtered through paper. The extraction was repeated with 10 volumes of the chloroform-methanol mixture. The filtrates were taken to dryness under reduced pressure. A small amount of absolute ethanol and a few drops of glacial acetic acid were added, and the extract taken to dryness again. The residue was extracted with chloroform and the extract transferred to a small weighed flask. The chloroform was removed under reduced pressure and the flask quickly weighed. Results are expressed as the percentage of lipid in the original liver.

RESULTS

The experimental groups are identified in table 2 according to the composition of the oil used to prepare the diet. The mean percentage of lipid in the livers of the animals that received the diet containing 10, 20 and 40% of the calories as fat are shown. The amount of liver lipid was not dependent to a significant degree upon the amount of fat in the diet. This is indicated by the mean lipid content of all diets supplying the three levels of fat and was confirmed by variance analysis.

The relative degree of unsaturation in the dietary fats was calculated from the oleic and linoleic acid content of the three oils used, these being the only unsaturated acids in these oils in appreciable amounts. The figures shown in the table are equal to the percentage of oleic acid plus twice the percentage of linoleic acid. As shown in figure 1, the amount of liver lipid appears to be inversely proportional to the logarithm of the degree of unsaturation of the dietary fat when the groups which received coconut oil alone are excluded. The correlation (excluding the groups receiving coconut oil alone) is excellent for the groups receiving the highest level of dietary fat ($r = -0.94$), somewhat less satisfactory for those receiving 20% of the calories as fat ($r = -0.81$) and relatively poor for lowest level of dietary fat ($r = -0.56$). The latter value barely reaches significance at the 5% level, whereas the other two are highly significant ($P < 0.001$). The slopes of the three lines shown in figure 1 are not significantly different.

The liver sections showed fine or large cytoplasmic vacuoles in the cord cells most

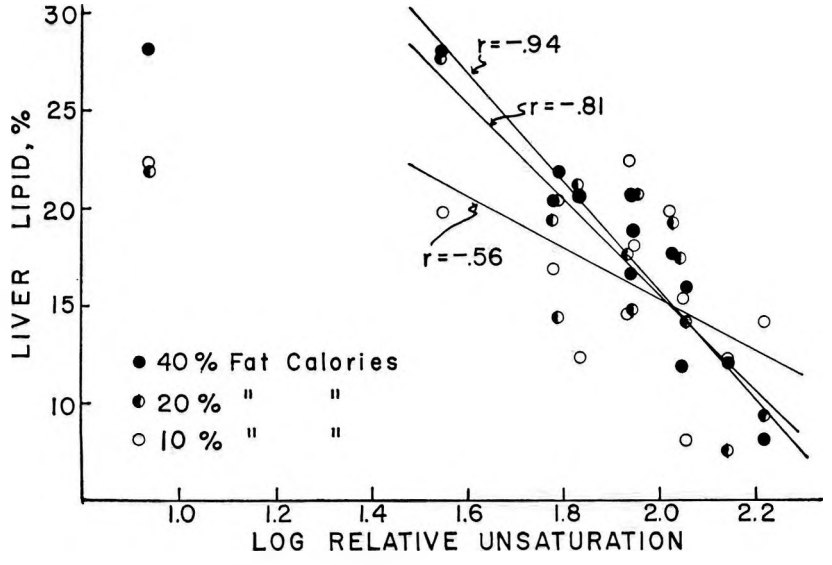

Fig. 1 The correlation between mean liver lipid and the log of the relative unsaturation of the dietary oils. Values at extreme left (coconut oil) not included in the calculations. Correlation coefficients and slope of the regression lines decreased with decreasing fat content of the diet.

prominently in the pericentral or paracentral regions. The livers of individual rats of some groups, e.g., 2, 3, 11, and 16, generally had no or only small amounts of visible fatty metamorphosis. Thus, these groups which had less than 10% of the wet liver as lipid showed a group fatty metamorphosis score of less than one plus. In contrast, groups having in excess of 20% of hepatic fat showed two to three plus hepatic fatty metamorphosis. Morphologic examination lacked the precision of chemical analysis in discriminating between groups showing only small differences in liver lipid. None of the kidneys showed an active "hemorrhagic kidney lesion" of choline deficiency. The kidneys of only three of the 155 rats showed an obvious gross healing or healed choline deficiency lesion. Microscopic examination, however, revealed that approximately one-fourth of the animals showed some evidence, generally slight, of renal damage. No abnormalities were observed in the myocardium or stomach.

At another time, a two-week experiment was conducted, using the same diets fed to groups 3, 6, 9, and 30. The percentage of liver lipid was 10.7, 15.7, 32.0 and 16.4 in these groups, respectively. Thus the results were similar to those shown in table 2.

Other groups of animals that received the diets described containing only safflower, olive and coconut oil and supplying 10 and 40% of the calories as fat, were allowed to continue for 7 weeks. At this time the percentage of liver lipid was 14.0, 13.9 and 15.7 for the three oils, respectively, at the low level, and 11.4, 22.4 and 28.6, respectively, at the high level. It appears therefore that after the animals consumed these diets for more extended periods, the level of dietary fat became a factor of significance. Furthermore, there appeared to be no difference in the lipogenic activity of the three oils when they were fed at the lower level.

Limited data are also available with the same three oils fed in diets containing only 8% casein and 35% fat over longer periods of time, 3 and 6 months. With these diets, lower in casein and higher in fat than the diets used here, the kind of dietary oil appeared to have little influence upon the amount of lipid deposited in the liver. These data are not reported in detail, but mentioned here to indicate that

in studies of this kind the results are clearly influenced by the kind of diet used and the length of the experiment.

Significant differences in the rate of growth of the animals in the various groups were also observed. The gain in weight varied from 23 gm in group 11 to 54 gm in group 23. Relatively poor growth was observed in groups 1 to 3 and groups 10 to 12, both of which received relatively large amounts of safflower oil. Groups 25 to 27 which also received considerable safflower oil did not show this growth depression. In view of the known relationship between food intake and the development of fatty livers, the possibility that gain in weight was a determining factor in the development of the fatty livers required consideration. Several lines of evidence apparently rule out this argument. The correlation coefficient between gain in weight and liver fat is poor ($r = 0.25$) and barely reaches significance at the 0.05 level of probability. Covariance analysis reveals highly significant effects of the dietary fat upon liver lipid not explained by the differences in growth. It is concluded that although the poor growth and limited food intake in certain groups may be of some significance, it does not offer an adequate explanation for the results obtained.

DISCUSSION

The conclusion reached in the principal experiment reported here is that, under the conditions and with the animals described, the accumulation of liver lipids was a function of the degree of unsaturation of the fat included in the diet. When relatively large amounts of fat were included in the diet there was a clear inverse relationship between the amount of liver lipid and the logarithm of the degree of unsaturation. The results were more variable when lower amounts of fat were included in the diet. The relationship did not, however, hold over the entire range of the dietary oils tested. Maximal liver lipid concentration was reached with the mixture composed of two-thirds coconut oil and one-third olive oil. This mixture was as lipogenic as coconut oil alone.

Channon concluded that the amounts of the saturated fatty acids in the dietary fat were positively related to the accumulation of hepatic lipid. This conclusion may be difficult to distinguish from those reached here when miscellaneous fats are studied, and the content of the mono- and polyunsaturated acids is not systematically varied. However, the difference in response when safflower oil was added to coconut oil as compared with the effect observed when olive oil was added, leaves little doubt that unsaturation was the primary factor influencing the accumulation of liver lipid.

It is clear that the diet used and the time that the experiments are allowed to continue may have pronounced effects upon the results obtained. Strain differences in animals and age are other likely variables of importance. Presumably these factors account for the differences observed in these studies and those reported by Best et al. ('58), for example.

In the preliminary analysis of the data, an explanation of the results was sought by the calculation of the multiple regression equation of the type:

$$\text{Liver lipid} = aS + bM + cP + d$$

where S, M and P are the amounts of the saturated, monounsaturated, and polyunsaturated fatty acids in the various diets expressed as percentages of the calories. This calculation yielded the equation:

$$(1)\quad \%\ \text{Liver lipid} = 0.58S + 0.11M - 0.70P + 16.71.$$

This equation indicates that saturated acids increase deposition of liver lipids and that the polyunsaturated acid lowers the deposition, whereas the monounsaturated acids have relatively little effect. The similarity of this equation to that developed by Keys et al. ('57) to describe the effects of oil composition upon serum cholesterol is evident. Of course, the conclusion that the deposition of liver lipids is inversely related to the degree of unsaturation also parallels the conclusion of Ahrens et al. ('57) that decreases in serum cholesterol in man are related to the degree of unsaturation of the dietary oils. Rigorous distinction between the two possibilities is difficult. In any event, it is possible that equation 1 may provide a good description of the data without being true in the operational sense that mono-

unsaturated acids are actually neutral in effect upon deposition of liver lipids or on serum cholesterol in man.

It seems likely that the changes in serum cholesterol level in man in response to dietary oils represent a change in liver function since the lowering of the cholesterol level is associated with an increased output of bile acids and sterols (Gordon et al., '57). The similarity in response of the liver lipids in the choline-deficient rat to the cholesterol response in man with changes in dietary fat suggests that some common factors are operative. Thus the delineation of the factors involved in the accumulation of liver lipid in the rat may be relevant to the important clinical problem of atherosclerosis.

SUMMARY

Under the conditions used, a two-week assay period and with a diet containing 12% casein, the accumulation of liver lipid in the choline-deficient rat appears to be inversely related to the degree of unsaturation of the dietary fat. The composition of the fat, rather than the amount in the diet, was the major factor influencing the amount of liver lipid. The results obtained are, however, dependent upon the basal diet used and the duration of the experiment.

The similarity of the conclusions reached in these studies with those relating dietary oils to serum cholesterol in man indicate that possibly similar factors are involved.

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Lipid Antioxidant Activity in Tissues and Proteins of Selenium-fed Animals[1]

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The interrelationship of selenium and vitamin E and their roles in animal nutrition has been of interest for several years (Schultze, '60). Since there is considerable evidence that vitamin E functions as an *in vivo* lipid antioxidant, the possibility that selenium functions by a similar mechanism was naturally considered. Very powerful selenium antioxidants are known,[2] and hence it is theoretically possible that selenium could form active lipid antioxidants even though fed at the very low level of 0.1 ppm.

Zalkin et al. ('60) showed that dietary selenite inhibited *in vivo* and *in vitro* lipid peroxidation in the vitamin E-deficient chick. Antioxygenic activity was found for various organic selenium compounds in model systems. Bieri ('61) and Bieri et al. ('61) reported that the addition of 0.14 to 1.4 ppm selenium to a vitamin E-deficient diet of chickens significantly reduced *in vitro* thiobarbituric acid values in homogenates of liver, kidney, and heart tissue. On the basis of this evidence and the known incorporation of selenium into tissue proteins, both groups suggested that dietary selenite formed lipid antioxidants, probably selenoamino acids and selenoproteins, in the animal. Olcott et al. ('61) showed that selenomethionine is a stronger lipid antioxidant than methionine and that it decomposes lipid peroxides. Selenium enters the sulfur metabolic pathway and is incorporated into proteins. For example, selenium incorporation into cytochrome c has been demonstrated by McConnell and Dallam ('62). In a study of the distribution of selenium in swine, Buescher et al. ('61) observed the kidney to reach the highest concentration when the liver contained the largest total of the 17 tissues sampled.

The purpose of this study was to determine whether liver and kidney selenoproteins possess antioxidants activity. Antioxidant activity is conveniently evaluated using reaction systems consisting of emulsified unsaturated lipid in aqueous buffer with hematin compounds as catalyst. In studies of vitamin E action the similarities of these reaction systems and animal tissues undergoing lipid peroxidation have been demonstrated (Tappel et al., '61; Lew and Tappel, '56).

EXPERIMENTAL

Animals received a nutritionally adequate basal diet supplemented with high but sub-toxic levels of selenite or selenate, as indicated in table 1.

High levels of selenium were fed for the biosynthesis of selenoproteins in amounts sufficient for fractionation and critical evaluation of antioxidant activity. The stock diets were nutritionally adequate in selenium and the control animals had sufficient selenium in their kidney and liver (table 1).

Groups of 5 mature, Sprague-Dawley strain, male rats were fed pulverized commercial laboratory chow,[3] with feed and water given ad libitum. Liver and kidney tissues from each group were pooled and stored frozen. Groups of 4, mixed crossbreed chickens received pulverized commercial growing mash. The kidney and liver were obtained from three mature ewes[4] that had received a basal alfalfa hay

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[2] Woodbridge, D. T. 1959 Oxidation, autoxidation, and antioxidant behavior of seleno-organic compounds. M.S. Thesis, University of London.
[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[4] Generously supplied by Dr. James O. Tucker, Head of the Division of Veterinary Science and Bacteriology, University of Wyoming, Laramie, Wyoming.

TABLE 1

Antioxidant activity of high-protein tissue fractions from selenium-fed animals

Animal	Selenium-fed		Tissues			Protective index	
	Form	Amount	Fraction no.	Protein	Selenium	5 Hours	10 Hours
		ppm		%	*ppm*		
			Kidney				
Chicken	none		1	97	0.5		
Chicken	selenate	10	2	100	18.0	1.4, 1.8	1.4
Chicken	selenite	10	3	95	16.5	1.1	1.1
Rat	none		4	84	0.2		
Rat	selenate	10	5	88	17.6	1.3, 5.3	1.3, 2.6
Rat	selenite	10	6	97	14.9	1.2, 1.3	1.3, 1.3
Sheep	none		7	82	0.5		
Sheep	selenate	14	8	98	24.4	5.8, 6.0	2.7
Sheep	selenate (0.01 mg/ 2.2 kg body wt/day)		9	100	22.8		
			Liver				
Chicken	none		10	99	0.5		
Chicken	selenate	10	11	84	14.9	1.6, 2.1	1.5, 1.6
Chicken	selenite	10	12	93	12.7	1.1, 1.5	1.2, 1.7
Rat	none		13	96	0.4		
Rat	selenate	10	14	95	17.4	1.0	1.0
Rat	selenite	10	15	93	14.6	1.0	1.2
Sheep	none		16	96	0.5		
Sheep	selenate	14	17	101	18.2	1.2	2.0
Sheep	selenate (0.01 mg/ 2.2 kg body wt/day)		18	100	17.6		

pelleted ration nutritionally adequate for reproduction and laction.

Lipid-free tissue fractions were prepared by repeated extractions of the tissue with chloroform-methanol followed by acetone. Purer protein fractions were obtained when these were dissolved in 0.5 M KCl, precipitated from nonprotein material with 5% trichloroacetic acid (TCA), centrifuged, washed with acetone and dried. Protein content was determined by the Biuret method or on acid hydrolyzed protein by the ninhydrin method of Troll and Cannan ('53). Yield of dry protein varied from 14 to 20%. Selenium contents were determined by the method of Handley and Johnson ('59) with minor modifications.

Oxygen absorption was measured manometrically at 37.7°C using 6-ml reaction vessels, an oxygen atmosphere, and mercury manometers. The reaction system consisted of 50 mg of dry tissue protein fraction, 0.5 ml of 33.3% linoleic acid[5] emulsion in 0.1 M phosphate buffer pH 7.2, and emulsifier.[6] The emulsion, protein, and hemoglobin were throughly mixed at the start and by rapid shaking at 180 strokes/minute during the reaction. Results are expressed as "protective indices" (PI) which are the ratios of the oxygen consumption of the control tissue fraction to that of the selenium tissue fraction.

Polarographic measurement of oxygen consumption was made by use of an oxygen electrode (Hamilton and Tappel, '63). The reaction system consisted of 10% methyl linoleate[7] and emulsifier;[8] these were emulsified in 0.1 M phosphate buffer adjusted to pH 7.2 containing 0.01 M KCl and the test substance. Rapid lipid peroxidation was initiated by adding 0.01 ml of 2×10^{-5} M hemoglobin. The PI values were calculated for 90% consumption of

[5] Nutritional Biochemicals Corporation, Cleveland.
[6] Tween 40.
[7] Pacific Vegetable Oil Corporation.
[8] Myrj 53.

dissolved oxygen by blank and test emulsions.

Synergism was similarly investigated by addition of α-tocopherol to both the control and selenium tissue emulsions. The following protective indices are calculated:

$$PI_{\alpha\text{-}T} = \frac{\text{Oxygen consumption control tissue}}{\text{Oxygen consumption control tissue} + \alpha\text{-tocopherol}}$$

$$PI_{Se} = \frac{\text{Oxygen consumption control tissue}}{\text{Oxygen consumption selenium tissue}}$$

$$PI_{Se + \alpha\text{-}T} = \frac{\text{Oxygen consumption control tissue}}{\text{Oxygen consumption selenium tissue} + \alpha\text{-tocopherol}}$$

If

$PI_{Se + \alpha\text{-}T} > PI_{\alpha\text{-}T} + PI_{Se}$, synergistic activity is indicated.

RESULTS AND DISCUSSION

Lipid antioxidant activity of tissues from selenium fed animals. Amounts of oxygen absorption of the peroxidizing lipid and rate changes as a function of time are shown in typical oxygen absorption curves in figure 1. From graphical representation of reaction data comparing control and tissue fractions such as those shown in figure 1, summarized data are shown in table 1. Some variation existed between duplicate reactions and the protective indices calculated at the end of 5- and 10-hour intervals of oxidation despite our efforts to standardize the method; this is shown by duplicate values in table 1.

Fraction numbers allow cross reference between tables and figures. Protein contents of the tissue fractions are all above 82%. Selenium contents are as anticipated if one considers the variables that affect selenium uptake and storage in tissues. Differences in selenium content of selenate and selenite probably reflect differences in feeding periods.

It is significant that definite antioxidant activity is exerted by kidney tissue fractions obtained from three widely different animals fed varying amounts of different

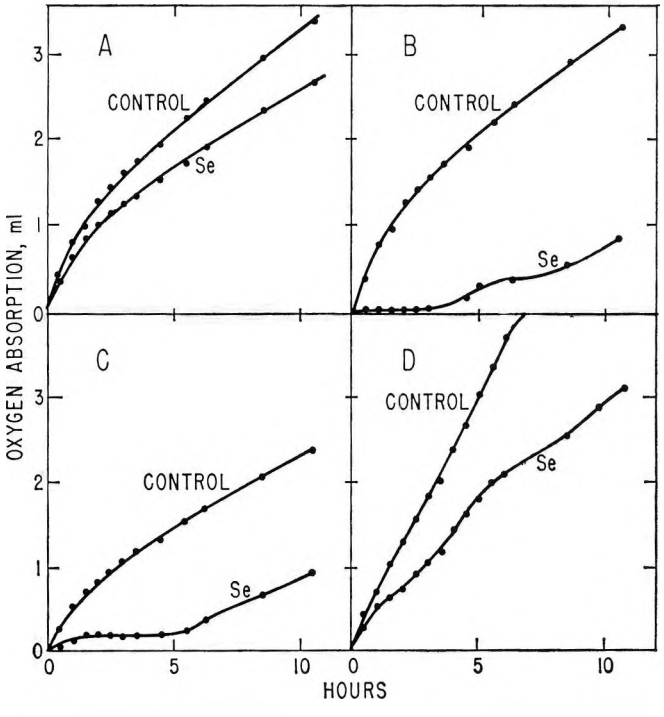

Fig. 1 Comparison of oxygen absorption of control and selenium-containing tissue fractions. A, control rat kidney, fraction 4, and selenite rat kidney, fraction 6; B, control chicken liver, fraction 10, and selenate chicken liver, fraction 11; C, control sheep kidney, fraction 7, and selenate sheep kidney, fraction 8; and D, control sheep kidney, fraction 7, and selenate sheep kidney, fraction 8, both with 1×10^{-4} M hemoglobin added.

selenium compounds (table 1). The liver tissue fractions of two of the animals contained relatively high levels of antioxidant activity. These three groups of animals exhibit different nutritional responses to extremely low as well as high levels of dietary selenium. Liver and kidney tissue are good sources of "Factor 3-selenium" (Schwarz, '61).

It appears likely that the antioxidant activity resides in selenoproteins since 82 to 100% of the tissue fractions are proteins. Additionally, a purified protein fraction contained a major fraction of the selenium and much of the antioxidant activity. The data in table 1 do not show any obvious relationship between antioxidant activity and selenium content of the tissue fractions. It is possible that only a portion of the selenium exists as a highly active compound or complex. Also, some of the protein in these fractions is insoluble in water and buffer and hence would exert little antioxidant effect.

Concentration of peroxides in the linoleate after manometric measurement of antioxidant activity was determined by the iodometric method and found to correlate with total oxygen absorption. There was no evidence for strong peroxide decomposition by the selenium antioxidants.

Polarographic measurement of antioxidant activity. The antioxidant activities of certain chicken and sheep kidney and liver tissue protein fractions were measured by a polarographic method. Some typical reaction curves are shown in figure 2.

Curves for the blank and control tissue systems are similar in shape and magnitude. Control tissue fractions consume oxygen at a more rapid rate than do the selenium tissue fractions throughout the experimental period. A twofold increase

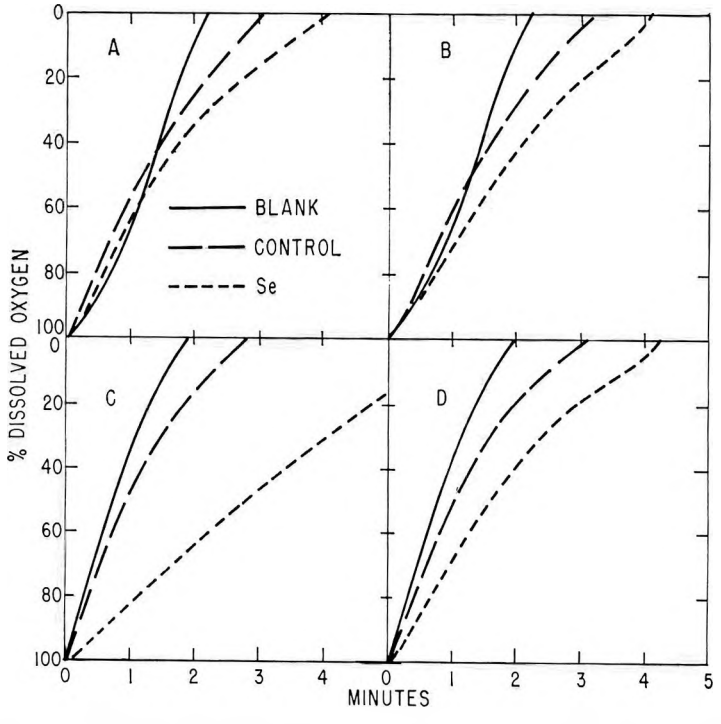

Fig. 2 Measurement of selenium antioxidants by polarographic determination of lipid peroxidation. Soluble fractions from 20 mg were added per ml of emulsion in A, B, and D. C was from 40 mg. A, control chicken kidney, fraction 1, and selenite chicken kidney, fraction 3; B, control chicken liver, fraction 10, and selenite chicken liver, fraction 12; C, control sheep kidney, fraction 7, and selenate sheep kidney, fraction 8; and D, control sheep liver, fraction 16, and selenate sheep liver fraction 17.

in the amount of tissue fraction does not markedly affect the oxidation rate of the control but greatly decreases that of the selenium tissue. The time required for reaction of 90% of the dissolved oxygen increases from 168 sec for 20 mg of tissue fraction to 275 sec for 50 mg. The differences in rate of oxygen uptake, between the control and selenium tissue fraction, become greater during the duration of the polarographic period and exist for several hours as is evident from manometric measurements shown in figure 1. This reaction system is a strongly oxidizing one that probably minimizes the antioxidant effects of a weak antioxidant such as the control tissue fraction that contains from 0.2 to 0.5 ppm of selenium.

The results obtained by the polarographic method are shown in table 2. It should be emphasized that the concentration of tissue fraction in the reaction system for polarographic determination is less than that present in the system used for the manometric determination. In the manometric determination the reaction system is 9% by weight of tissue fraction, whereas in the polarographic determination the reaction system is approximately 0.5 to 1% soluble tissue fraction. The polarographic method measures the antioxidant effect during the first few minutes of the reaction whereas the manometric technique measures the oxygen consumption for 10 hours.

The protective index (table 2) of 10 mg/ml of the acid-hydrolyzed selenate chick kidney fraction is higher than that for 20 mg/ml of the original fraction. This indicates that the antioxidant activity is increased by solubilizing more of the selenium compounds or changing the original compounds or complexes into a more active form. It is possible that the compounds or complexes exerting the antioxidant effect of our hydrolyzed chicken kidney fraction are similar to the "Factor 3-selenium" of Schwarz ('61) since it was prepared in the same manner as "Factor 3."

Polarographic studies indicate that a concentration of 1×10^{-3} M (2 μmoles) α-tocopherol exerts approximately the same antioxidant effect as the soluble extract of 20 mg of the selenium animal tissue fraction containing approximately 15 ppm selenium (4×10^{-3} μmoles). Assuming that all of the active selenium compounds are soluble and contain 1 Se atom each, the relative effectiveness of selenium to α-tocopherol on a molar basis is 500 to 1. This indicates a very powerful biological antioxidant.

Effect of hemoglobin addition to the reactions measured manometrically. As myoglobin and hemoglobin are known to be powerful biocatalysts for lipid peroxidation and as the amounts present in tissue fractions probably varied within rather wide limits, excess of hemoglobin was added to some of the reaction systems to test whether differences in amount of these catalysts influenced our results. Addition of hemoglobin usually increased oxy-

TABLE 2

Antioxidant activity of high-protein tissue fractions from selenium-fed animals determined by polarography

Animal	Tissue fraction no.	Portion of fraction	Tissue fraction	Protective index
			mg/ml emulsion	
		Kidney		
Chicken	2	soluble	20	1.4
Chicken	2	hydrolysate, acid	10	2.4
Chicken	2	entire	20	1.3
Sheep	8	soluble	20	1.2
Sheep	8	soluble	40	2.2
		Liver		
Chicken	11	soluble	20	1.6
Sheep	17	soluble	20	1.3
Sheep	17	soluble	20	1.3
Sheep	17	soluble	40	1.7

gen consumption and sometimes changed the kinetics of the reaction as evidenced by comparison of the curves of C and D in figure 1. But it did not greatly affect the calculated protective indices as shown by a comparison of the summarized data in table 3 with that of table 1.

TABLE 3

Antioxidant activity of selenium-tissue fractions with added lipid peroxidation catalyst[1]

Sheep tissue	Tissue fraction no.	Protective index	
		5 Hours	10 Hours
Kidney	8	1.5, 1.8, 2.1	1.6, 1.8, 2.2
Kidney	9	2.4	2.6
Liver	17	1.5, 2.1	1.5, 2.1
Liver	18	2.2	2.1

[1] 1×10^{-4} M added hemoglobin.

It should be emphasized that the presence of relatively large amounts of either the lipid or hemoglobin or both can overpower a weak antioxidant but exert little effect upon these strong selenium antioxidants.

Effect of adding known antioxidants. The effect of the addition of known amounts of weak antioxidants to both the control and selenium tissue fractions was investigated; the data are presented in table 4.

The protective index was usually somewhat lower in the presence of ascorbic acid or α-tocopherol. When both ascorbic acid and glutathione were added to the system the protective index was much lower. The action of added glutathione might be related to selenite catalysis of the oxidation of glutathione and other sulfhydryl compounds (Tsen and Tappel, '58). These results indicate that these antioxidants and selenium antioxidants function independently.

Antioxidant activity of tissue protein fractions. Results previously discussed indicate that the selenium sheep kidney fraction has the highest selenium content and possesses the greatest antioxidant activity. To provide further evidence of the intimate relationship between the selenoprotein content and antioxidant activity, a KCl-soluble, TCA-precipitated protein fraction was prepared from the control and selenium kidney tissue fractions (table 5).

These protein fractions, numbers 19 and 20, had a higher protein content and also a higher selenium content than the insoluble residue; however, the selenium content of each was less than that of the original fraction. This indicates that some of the selenium remained in the protein-free supernatant and was discarded. No attempt was made to determine the selenium content or antioxidant activity of this supernatant. The protein fraction 20 contains much of the antioxidant activity. Antioxidant activity was also found in the insoluble residue fraction 22. The antioxidant activity of the purified protein is exhibited when hemoglobin alone or with α-tocopherol is added to the system. The possible synergistic action of the selenoprotein fractions and α-tocopherol will be discussed later. That antioxidant activity can be extracted by a known protein solvent and precipitated by a recognized protein precipitant to yield a protein fraction which exhibits high antioxidant activity is important. Since this protein fraction contains a fairly high content of selenium, the results indicate that

TABLE 4

Antioxidant activity of selenium-tissue fractions with added antioxidants

Animal	Tissue fraction no.	Antioxidant	Molar concentration in system	Protective index[1]	
				5 Hours	10 Hours
Chicken liver	11	ascorbic acid	1×10^{-3}	1.3	1.2
Chicken liver	11	ascorbic acid + glutathione	1×10^{-3}	1.0	1.1
Sheep kidney	9	α-tocopherol	2×10^{-3}	1.4	1.4
Sheep kidney	8	α-tocopherol	5×10^{-3}	1.2	1.4
Sheep liver	18	α-tocopherol	2×10^{-3}	1.5	1.8
Sheep liver	17	α-tocopherol	5×10^{-3}	7.6	1.6

[1] Protective index = $\frac{\mu l\ O_2 \text{ control tissue + antioxidant}}{\mu l\ O_2 \text{ selenium tissue + antioxidant}}$.

TABLE 5

Antioxidant activity of protein and tissue fractions from sheep kidney

Fraction	Fraction no.	Protein	Selenium	Protective index	
				5 Hours	10 Hours
		%	*ppm*		
Chloroform-methanol and acetone-extracted tissue	7	82	0.5		
	8	98	24.4	5.8, 6.0	2.7
TCA[1] precipitate of KCl extract of fractions 7 and 8	19	99	0.4		
	20	99	15.2	2.6	4.0
Insoluble residue from KCl extraction of fractions 7 and 8	21	96	0.4		
	22	96	10.5	0.9, 1.2	1.3, 1.4
TCA[1] precipitate + 1×10^{-4} M hemoglobin	19	99	0.4		
	20	99	15.2	2.0	2.0
TCA[1] precipitate + 1×10^{-4} M hemoglobin + 2×10^{-3} M α-tocopherol	19	99	0.4		
	20	99	15.2	1.8	2.3

[1] 5% Trichloracetic acid (TCA) concentration.

TABLE 6

Antioxidants in linoleic acid emulsion systems containing protein

Molar concentration α-tocopherol	Tissue	Tissue fraction no.	Protective index[1]	
			5 Hours	10 Hours
Caseinate + 1×10^{-4} M hemoglobin				
1×10^{-4}			0.6	0.7
1×10^{-3}			1.3	1.1
2×10^{-3}			1.6	1.7
4×10^{-3}			2.3	2.4
5×10^{-3}			2.8	2.8
1×10^{-2}			5.4	6.0
Control sheep tissue protein fractions + 1×10^{-4} M hemoglobin				
2×10^{-3}	kidney	7	1.3	1.2
5×10^{-3}	kidney	7	1.6	1.1
2×10^{-3}	liver	16	1.4	1.3
5×10^{-3}	liver	16	1.5	1.5
2×10^{-3}	kidney	21	1.4	1.8

[1] Protective index = $\frac{\mu l\ O_2 \text{ uptake of caseinate or tissue protein fraction}}{\mu l\ O_2 \text{ uptake of caseinate or tissue protein fraction + antioxidant}}$.

the powerful antioxidant is a selenoprotein.

Antioxidant activity of α-tocopherol and other substances. The tocopherols have been shown by Lew and Tappel ('56) and Tappel et al. ('61) to be weak antioxidants when added to *in vitro* model systems. As the principal role of vitamin E in animal nutrition is that of an *in vivo* antioxidant, direct comparisons of the selenium antioxidants studied here with α-tocopherol is important. To more closely approximate *in vivo* conditions the effect of α-tocopherol addition to the model system containing either control or selenium tissue fractions was also investigated.

The results in table 6 indicate that addition of 1×10^{-3} M to 2×10^{-3} M α-tocopherol to the caseinate or tissue protein gives approximately the same PI (1.3 to 1.6) as the mean PI of the selenium protein fractions (tables 1 and 3).

Comparing the antioxidant activity of the selenium present in the tissue frac-

tions with α-tocopherol indicates that on a molar basis it possesses 50 to 100 times the antioxidant activity of α-tocopherol.

It is appropriate to compare the antioxidant activities determined by both polarographic and manometric methods with the known biological effectiveness of dietary selenium compounds and their deposition in animal tissue. As a dietary component selenium fed as selenite is 500 to 1000 times as active as α-tocopherol on a weight basis in prevention of exudative diathesis in the chick (Schwarz et al., '57). On a mole basis it is up to 200 times as active. Similarly for liver necrosis in the rat, selenium is 500 times as active as α-tocopherol on a weight basis or 100 times on a mole basis. Using the mean selenium content of lamb tissue as 2.2 ppm for kidney and 0.28 ppm for liver (Hartley and Grant, '61) and assuming an average tocopherol of 10 ppm, the mole ratios of selenium to tocopherol are 1:1 for kidney and 1:6 for liver. Other comparisons (Zalkin et al., '60) also have indicated that selenium and tocopherol are deposited in comparable amounts with ratios of selenium:tocopherol of 1:3 to 1:10. These comparisons indicate that the selenium antioxidants studied here have more than sufficient activity compared with tocopherol (50 to 500 times) to account for the quantitative differences in dietary requirements and tissue contents.

Synergistic action between selenium and α-tocopherol. Since both selenium and vitamin E appear to function as antioxidants, they probably have an *in vivo* interrelationship and may perhaps exert synergistic effects. The results of our research to detect possible synergism of α-tocopherol and the selenium present in the animal tissue fractions are shown in table 7.

The protective index for α-tocopherol is shown by sheep tissue plus α-tocopherol. That for selenium is given by the selenium-sheep tissue, and the protective index for α-tocopherol and selenium is the selenium sheep tissue plus α-tocopherol. If the value of the latter is larger than the sum of the first two, strong synergism is indicated. The results show that there is no strong synergism between α-tocopherol and the selenium antioxidant; in only three instances was the latter value greater. Slight synergistic activity is indicated with the purified protein fraction.

Mechanisms of selenium antioxidants. We have considered the possible mechanisms for the selenium antioxidants both from knowledge of the analogous sulfur compounds and available information on selenium compounds. The main possibility indicated in this research is that

TABLE 7

Evaluation of possible synergism of selenium and α-tocopherol

Sheep tissue	Tissue fraction no.	Molar concentration α-tocopherol	Protective index	
			5 Hours	10 Hours
Kidney	7	2×10^{-3}	1.3	1.3
Kidney — Se	9	0	1.7	1.8
Kidney — Se	9	2×10^{-3}	1.8	2.1
Kidney	7	5×10^{-3}	1.6	1.1
Kidney — Se	8	0	1.5	1.6
Kidney — Se	8	5×10^{-3}	1.9	1.6
Liver	16	2×10^{-3}	1.4	1.3
Liver — Se	18	0	2.2	2.1
Liver — Se	18	2×10^{-3}	2.2	2.3
Liver	16	5×10^{-3}	1.2	1.1
Liver — Se	17	0	1.5	1.6
Liver — Se	17	5×10^{-3}	9.1	1.7
Kidney	21	2×10^{-3}	1.6	1.5
Kidney — Se	22	0	2.0	2.0
Kidney — Se	22	2×10^{-3}	2.9	3.5

selenium antioxidants act as primary antioxidants like α-tocopherol, reacting with the free radical intermediates of lipid peroxidation thereby breaking the chain reaction.

Thus, in the chain reaction,

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot} \quad (1)$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \quad (2)$$

the hydroperoxyl radical, $ROO^{\cdot}$, is a propagating agent and because of the fast reaction of $R^{\cdot}$ with O_2 it is the major radical present in the steady state.

Selenium compounds of the general types R′SeH and R′SeR′, could react to remove this radical:

$$ROO^{\cdot} + R'SeH \rightarrow ROOH + R'Se^{\cdot} \quad (3)$$

$$ROO^{\cdot} + R'SeR' \rightarrow R'\overset{\overset{O}{\|}}{S}eR' + ROH + ? \quad (4)$$

The $R'Se^{\cdot}$ of reaction (3) should be considerably more stable than analogous sulfur compounds and could either combine,

$$2R'Se^{\cdot} \rightarrow R'SeSeR' \quad (5)$$

or react with sulfur compounds which are present in larger quantity:

$$R'Se^{\cdot} + R''SH \rightarrow R'SeH + R''S^{\cdot} \quad (6)$$

Other intriguing possibilities include lipid peroxide decomposition; direct evidence for this reaction comes from studies of the antioxidant properties of selenomethionine (Olcott et al., '61):

$$ROOH + R'\overset{\overset{O}{\|}}{S}eR' \rightarrow ROH + R'SeR' \quad (7)$$

$$ROOH + 2R'SeH \rightarrow ROH + R'SeSeR' + H_2O \quad (8)$$

or for interaction with sulfur compounds:

$$ROOH + R'SeH + R''SH \rightarrow ROH + R'SeSR'' + H_2O \quad (9)$$

Synergism with α-tocopherol (α-TH) though small could arise by reduction of α-tocopherol radical

$$\begin{array}{ccc} ROO^{\cdot} & \overset{\alpha\text{-TH}}{\searrow\!\!\swarrow} & R'Se^{\cdot} \\ ROOH & \underset{\alpha\text{-T}^{\cdot}}{\nearrow\!\!\searrow} & R'SeH \end{array} \quad (10)$$

Reactions analogous to (3) (5) (6) (8) (9) are known mainly from the reactions of peroxide with thiol compounds and the analogous selenium compounds and the radiation chemistry of thiol compounds (Markakis and Tappel, '60). Reactions analogous to (4) and (7) for organic peroxides and organic sulfides are reviewed by Barnard et al. ('61).

To advance our knowledge of the selenium antioxidants, their composition and mechanisms of action, more study of simpler selenium compounds such as the selenoamino acids is needed.

SUMMARY

Lipid antioxidant activity of lipid-free tissue fractions, from control and selenium-fed chickens, rats, and sheep was studied. Animals were fed 10 or 14 ppm of selenium supplied either as sodium selenite or sodium selenate added to a nutritionally adequate basal ration. Antioxidant activities of the tissue fractions were tested by manometric and polarographic techniques. The protein contents of the tissue fractions varied from 82 to 100% and the selenium-fed animal tissue fractions contained levels of selenium ranging from 13 to 24 ppm. Antioxidant activity expressed as protective indices varied from 1.0 to 6.0 indicating antioxidant activity for all selenium kidney and liver fractions tested except rat liver. Kidney fraction from sheep contained the highest antioxidant activity. Addition of hemoglobin to the manometric system increased the total oxygen consumption but did not greatly influence the protective indices of the selenium tissue fractions. Addition of α-tocopherol to the control and selenium tissue fractions decreased the total oxygen consumption, did not greatly affect the protective indices, and appeared to exert its antioxidant effects largely independent of the selenium antioxidant. Antioxidant activity was shown to be associated with the tissue selenoproteins. Selenium antioxidants were found to possess 500 times the antioxidant activity of α-tocopherol by the polarographic method and 50 to 100 times in the manometric method. Synergism between α-tocopherol and the tissue selenium was not large. Mechanisms to explain the antioxidant action of selenium in the animal body are presented.

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Metabolism and Biological Activity of Vitamin A Acid in the Chick

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In the rat vitamin A acid replaces vitamin A alcohol for growth functions but not for the visual process (Dowling and Wald, '60). Thompson et al. ('61a, b) have reported that the acid will not maintain normal fertility in female rats nor spermatogenesis in males. No storage of the acid or any known form of vitamin A has been detected in animal tissues even after large doses were fed or injected (Arens and Van Dorp, '46; Redfearn, '60). Recently, Jurkowitz ('62) found vitamin A acid in human plasma after its ingestion. In the present report, unchanged vitamin A acid is shown to be present in tissues of chicks shortly after its oral administration. Using C^{14}-labeled vitamin A acid, the transient nature of the compound is confirmed and metabolites or degradation products are shown to occur in liver.

EXPERIMENTAL

One-day-old chicks of mixed sex were given a purified, vitamin A-free diet consisting in per cent of isolated soybean protein,[2] 30; cottonseed oil,[3] 4; salt mix (Fox and Briggs, '60), 6; vitamin mix (Fox et al., '55), 0.2; L-cystine, 0.3; DL-methionine, 0.2; and glucose monohydrate, 59.3. Groups of 7 to 9 were housed in electrically heated brooders with raised wire floors, and water and food were supplied ad libitum. Clear, aqueous dispersions of crystalline vitamin A acid[4] or vitamin A acetate[5] were prepared by dissolving the compound in ethanol, adding polyoxyethylene sorbitan monooleate (Tween 80) and then water so that the final solution contained 20% ethanol and 10% Tween. Ethoxyquin[6] at a concentration of 0.1% was included as antioxidant. The solutions were refrigerated and prepared fresh every 5 days. Periodic chemical analyses indicated no significant loss of potency in such solutions up to 14 days. A standard dose of 0.25 ml was given daily by a syringe with a long, blunt needle introduced into the crop.

After dosing chicks with vitamin A acid, the tissues (except plasma) were homogenized in water and an equal volume of 95% ethanol added. The mixtures were then extracted with diethyl ether. Measurement of vitamin A acid in the tissue extracts was made with antimony trichloride at 573 mμ.

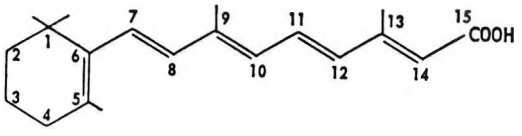

Fig. 1 Structure of all-*trans* vitamin A acid.

C^{14} vitamin A acid labeled in the 6 and 7 positions (fig. 1) with a specific activity of 19.9 μc/mg was used.[7] Paper chromatography and subsequent scanning, as described below, showed that all radioactivity was located in the zone of vitamin A acid as identified by spraying with antimony trichloride. Solutions for administration to chicks were prepared as described above for unlabeled vitamin A acid.

For counting, ether or hexane solutions were evaporated in a planchet containing

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[2] ADM Assay Protein C-1, Archer-Daniels-Midland, Minneapolis, Minnesota.

[3] Wesson Oil. The Wesson Oil Company, New Orleans, Louisiana.

[4] Eastman Organic Chemicals, Rochester, New York.

[5] See footnote 4.

[6] Santoquin, Monsanto Chemical Company, St. Louis, Missouri.

[7] Kindly supplied by Dr. O. Isler of Hoffmann-La Roche, Basle.

a circle of lens tissue (Entenman et al., '49). Thirty microliters of a 4% alcoholic solution of Tween 80 were added and the solvent evaporated to facilitate uniform spreading for counting at infinite thinness using a windowless gas-flow counter.

Paper chromatographic separation of the various forms of vitamin A was carried out with zinc carbonate-impregnated Whatman no. 1 filter paper (Green et al., '55). The ascending technique was performed with strips 18 × 150 mm in large test tubes at room temperature. The solvent system was 1:1:38 (by volume) of glacial acetic acid, absolute ethanol and cyclohexane. Lipid extracts from tissue samples as large as one gram could be applied in this manner without interfering with the separation. The zones located by ultraviolet light were cut out and eluted with ethanol-diethyl ether (1:2). After washing and drying the eluate the antimony trichloride reaction was applied quantitatively to each fraction. Recovery experiments with 0.5 to 5 μg of vitamin A alcohol, aldehyde or acid showed that losses of each of the three compounds in the chromatographic procedure did not exceed 10%. The average R_f values were 0.29, 0.63 and 0.94 for the acid, alcohol and aldehyde, respectively. Esters of vitamin A alcohol migrated with the solvent front. Radioactive zones were located by use of a strip scanner and recorder.

RESULTS

Biological activity. Vitamin A deficiency symptoms in control chicks developed consistently in 14 to 17 days, and were first apparent as an unsteadiness which progressed to a marked ataxia in two to three days. Paralleling the ataxia was a noticeable enlargement of the third eyelid which gave the appearance of the eye being displaced toward the back. All untreated chicks died by the 28th day.

Preliminary experiments showed that the response of depleted chicks to vitamin A acid depended upon the length of time they had been fed the deficient diet before symptoms developed in the negative control chicks. This suggested a sparing action of vitamin A acid on the residual body stores of vitamin A. When dosing was started on the 14th day, by which time no vitamin A was found in the livers by chemical analysis, relatively large doses of vitamin A acid were required to prevent all symptoms. Table 1 shows data from a representative experiment. Ten micrograms daily of vitamin A alcohol produced optimal growth and prevented all symptoms. With the acid, good growth was obtained with 10, 20 or 40 μg, but even the highest dose would not prevent symptoms in all birds. Of particular interest was the repeated observation that whereas 10 to 40 μg of the acid daily always yielded a significant number of birds with marked unsteadiness of gait, the growth rate of such birds was similar to that of chicks without symptoms.

Autopsy of chicks given low daily doses of vitamin A acid, 2.5 to 10 μg, for 4 to 5 weeks, revealed no gross abnormalities including an absence of urates in the kidneys and ureters, even though such birds had marked neurological symptoms. It became apparent throughout these studies that the central nervous system is the most vulnerable tissue in vitamin A deficiency and that apparently minor lesions

TABLE 1

Biological activity of vitamin A acid in the chick

Daily dosage[1]		Avg wt 5 weeks	No. survivors/ no. started	No. ataxic	No. with eye symptoms
μg		*gm*			
2.5	Vitamin A alcohol[2]	302	1/8	5	4
5	Vitamin A alcohol[2]	298	7/8	7	0
10	Vitamin A alcohol[2]	354	8/8	0	0
5	Vitamin A acid	296	7/8	7	4
10	Vitamin A acid	345	7/8	2	6
20	Vitamin A acid	349	7/7	2	2
40	Vitamin A acid	361	8/8	1	3

[1] Given orally from the 10th day.
[2] As vitamin A acetate.

are irreversible even by vitamin A alcohol. Comparison of testes weights in two groups receiving 10 μg vitamin A alcohol or 20 μg vitamin A acid daily for 28 days, revealed no significant difference (25.8 ± 1.4 and 22.8 ± 1.9 mg/gm body weight, respectively).

In two experiments where 100 μg of vitamin A acid were given daily starting on the 9th day, and continued for 6 weeks, several birds always had hypertrophied third eyelids, with occasional conjunctival infections. During the last week of these experiments it was noted that birds often exhibited slight ataxia in the morning prior to dosing, but that by afternoon no unsteadiness was evident. This led to the realization, supported by results from the metabolic studies with C^{14} vitamin A acid, that single daily doses of even 100 μg were so rapidly metabolized in the body that the duration of effect was less than 24 hours. When vitamin A acid was added directly to the diet (6 mg/kg) so that a more or less continuous supply was obtained both day and night, then chicks developed normally without any evidence of ataxia or eye symptoms. Histological examination of eyes from chicks given 50 μg of vitamin A acid daily for 6 weeks revealed no differences from comparable eyes of control chicks getting vitamin A alcohol.

An experiment was designed to confirm the observation mentioned above that vitamin A acid spares vitamin A already in the body. Daily dosing of groups of chicks fed the deficient diet was begun on the 7th, 12th or 16th days and the time of onset of symptoms noted (table 2). The data illustrate clearly that the earlier the supplementation was begun the longer was the delay in appearance of symptoms. This effect could not be explained by storage of vitamin A acid, as will be evident from the results of experiments on the metabolism of the acid.

Metabolism of vitamin A acid. Analyses of liver and plasma from chicks that had received 50 or 100 μg of vitamin A acid daily for three weeks revealed no evidence of either vitamin A acid or alcohol. When vitamin A-depleted chicks were dosed orally with a relatively large amount of the acid and killed after 3, 6 or 18 hours, vitamin A acid could be found in the tissues (table 3). A significant quantity of the acid was present at three hours in the plasma, liver and intestinal wall. These values had decreased considerably by 6 hours and by 18 hours only traces were detectable in the liver and intestine.

TABLE 2

Sparing action of vitamin A acid in delaying the onset of deficiency symptoms

First day of supplement[1]	No. symptoms at day				Avg wt 4 weeks
	17	21	24	30	
					gm
7	0	0	0	2	322
12	0	0	3	7	311
16	0	2	5	6	300
16 (vitamin A acetate)	0	0	0	0	345
None	4	8	8	8	—

[1] 20 μg vitamin A acid orally daily, 8 chicks/group. Symptoms were ataxia and eye infections.

TABLE 3

Absorption and distribution of vitamin A acid in the chick[1]

Time	Plasma	Liver	Excreta	Intestines		
				Mucosa	Muscle	Contents
hours	*μg/ml*	*μg/gm*	*μg*	*μg/gm*	*μg/gm*	*μg*
3	13.0	15	—	10	6	119
6	2.0	3	275	6	3	42
18	0	0.6	425	1.8	2.3	18

[1] Average values for three chickens for each time interval; 3 mg vitamin A acid in water dispersion containing Tween 80 and ethoxyquin given orally to fasted chicks weighing 180 to 250 gm.

TABLE 4

Intracellular distribution of vitamin A acid in chicken liver[1]

Cell fraction	Vitamin A acid content	
	μg/gm	%
Whole homogenate	13.0	100
Nuclei	1.3	10.0
Mitochondria	1.3	10.0
Microsomes	8.7	67.0
Supernatant	3.3	24.0

[1] 3 mg of vitamin A acid was given orally and the chick killed after 3 hours; 3 gm of liver was homogenized in 0.25 M sucrose and fractionated by centrifugation (Schneider and Hogeboom, '50).

Paper chromatography of these extracts revealed that vitamin A acid was the only antimony trichloride-reacting substance present. Evidence that the material in the liver was unchanged vitamin A acid was provided by the absorption curve obtained on eluates from several livers. The maximum was at 343 to 348 mμ in cyclohexane.

The distribution of vitamin A acid in the liver is shown in table 4. The microsomal fraction contained 67% of the total vitamin A acid. This is in marked contrast with the predominant localization of vitamin A esters in the supernatant fraction (Krishnamurthy et al., '58).

Experiments with C^{14} vitamin A acid. The labeled compound (fig. 1) was diluted with cold vitamin A acid so that 1 μg was equivalent to 1000 disintegrations per minute (dpm). A dose of 600 μg (600,000 dpm) was given orally to vitamin A-deficient chicks which were killed after 1, 3, 6 and 18 hours. Tissues were removed and frozen until analyzed. Four different fractions were extracted from the homogenized tissues (see footnote, table 5).

The radioactivity extracted directly by ether (fraction A) or by ether after addition of ethanol (fraction B) was found to be due to unchanged vitamin A acid. This was shown by co-chromatography with unlabeled vitamin A acid on zinc-carbonate impregnated paper. The activity in fractions A and B as detected by the strip-scanner coincided exactly with the color produced by antimony trichloride. The activity extracted after acidifying the homogenate (fraction C) stayed at the origin of the paper and the material did not give any color with antimony trichloride. No information was obtained as to the possible chemical nature of this material

TABLE 5

Distribution in chick tissues of radioactivity from a single dose of vitamin A acid[1]

Time	Plasma				Liver				Intestinal mucosa				Intestinal muscle				Intestinal contents				Excreta			
	Fraction[2]				Fraction				Fraction				Fraction				Fraction				Fraction			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
hours																								
1	2.8	1.2	0.6	0.5	3.2	4.8	1.2	0.9	3.0	5.9	1.9	2.3	1.5	1.0	0.9	0.5	8.4	11.1	2.4	2.5	—	—	—	—
3	0.8	0.6	0.4	0.2	2.0	2.1	0.3	0.4	4.7	7.6	2.1	1.8	2.2	3.2	1.8	2.1	10.5	9.5	8.7	27.5	10.0	12.0	5.8	8.2
6	0.5	0.5	0.5	0.4	0.8	0.7	0.4	0.5	3.2	4.0	3.0	2.3	1.7	2.2	1.8	1.9	4.8	4.6	4.0	18.2	38.0	40.0	8.2	38.0
18	0.2	0.1	0.4	0.4	0.4	0.6	0.4	0.9	0.4	0.5	1.8	2.0	0.2	0.2	0.7	1.2	—	—	—	—	81.8	51.8	19.8	52.8

[1] 600 μg of C^{14} vitamin A acid = 600,000 dpm given orally to chicks fasted for 24 hours. One chick at each time interval; dpm (in thousands) expressed per gram of tissue or per milliliter of plasma except excreta, which is total for each time period.

[2] Aqueous homogenates were extracted with 3 volumes of diethyl ether (fraction A). An equal volume of 95% ethanol was added to the aqueous layer and re-extracted with ether (fraction B). The aqueous layer was acidified with 0.1 N HCl to pH 2 and again extracted with ether (fraction C). The aqeous supernatant after centrifuging to remove the protein was counted as fraction D.

nor of activity remaining in the water phase (fraction D).

Table 5 shows the distribution of activity in these 4 fractions in blood, liver and the intestinal tract. These were the only tissues that had a significant amount of activity. The highest concentrations of the acid were reached in the blood plasma and liver after only one hour. Absorption by the intestine appeared to continue at about the same rate at 1, 3 and 6 hours. In general, these data for unchanged vitamin A acid parallel the results using the unlabeled compound (table 3). Of special interest was the rapid appearance of the unknown products in fractions C and D. The marked increase in these substances in the intestinal contents from 1 to 3 or 6 hours indicate that much of the modification in the molecule occurs in the intestinal lumen. Although the radioactivity in the tissues was very low at 18 hours, the combined amount of these two metabolic products exceeded the amount of vitamin A acid. At this time, approximately one-third of the activity given was recovered in the excreta. No attempt was made to account quantitatively for the whole dose; the stomach, crop and esophagus were not analyzed and undoubtedly much activity was in these tissues, especially after one and three hours. Five days after administration of the C^{14} vitamin A acid, no radioactivity could be detected in the blood or liver, indicating that the acid and its metabolites are no longer present in the body.

Biological activity of radioactive fractions prepared from chicken liver. Chromatography, ultraviolet spectra and the antimony trichloride color reaction showed that the radioactivity due to fractions A and B from tissue extracts was due to unchanged vitamin A acid, whereas C and D were unknown metabolites. Experiments were conducted to determine the possible biological activity of the various fractions. To prepare enough of these materials for biological testing, depleted chicks were given orally 10 mg vitamin A acid diluted with a small amount of C^{14} acid (1 μg = 27 dpm). After 18 hours, the birds were killed, the livers removed, homogenized in water and extracted as described (table 5). Fractions A and B (pooled) and fraction C (separately) were evaporated to dryness and prepared for feeding in aqueous dispersion with 10% Tween 80, 20% ethanol and 0.1% ethoxyquin as antioxidant (0.5 ml equivalent to 2 μg vitamin A acid on the basis of radioactivity). Experiments showed that if a homogenate of liver from chicks dosed with C^{14} vitamin A acid was dialyzed for 24 hours against water, a considerable amount of radioactivity appeared in the dialysate which corresponded to the radioactivity in fraction D. Fraction D for biological testing was therefore prepared by concentrating *in vacuo* at 50°C the dialysate from 10 livers from chicks dosed with C^{14} vitamin A acid. The dialysate was lyophilized and yielded 10 gm of powder which contained radioactivity equivalent to 120 μg of vitamin A acid. This was dissolved in water.

Weanling male rats of the Holtzman strain were fed a purified-type casein diet devoid of vitamin A. When they reached a weight plateau (28 to 30 days), daily doses of the above liver fractions were given by stomach tube. Three or four rats were used for each test solution. In the first experiment, the water-soluble metabolite from liver (fraction D) was completely without biological activity when a dose equivalent to 3 μg of vitamin A acid was given daily for 10 days. Control rats receiving 3 μg of the acid responded with a marked weight gain. In the second experiment, the combined fractions A and B (unchanged vitamin A acid) and fraction C were tested at a dose of 3 μg daily. Whereas the rats receiving combined fractions A and B maintained weight or gained slowly, comparable to rats receiving 2 μg of vitamin A acid, the rats receiving fraction C equivalent to 3 μg of the acid, lost weight steadily. In contrast to the untreated control animals that lost weight and developed severe eye symptoms during the 10-day test period, the rats getting fraction C had normal eyes. In the third experiment, doses equivalent to 5 μg of vitamin A acid were tested. Combined fractions A and B, which contained a large amount of lipid, produced erratic results in that only 1 of the 4 rats gave a growth response. With fraction C, 2 of the 4 rats responded positively, whereas 3 of 4 ani-

mals given vitamin A acid gained in weight (all untreated controls decreased).

In view of the crude nature of these various fractions these results must be considered tentative. It appears, however, that the water-soluble metabolite(s) in fraction D has no biological activity, but that the fat-soluble metabolite in fraction C, which does not react with antimony trichloride, does have activity, particularly with respect to prevention of eye symptoms.

DISCUSSION

The combined evidence from the experiments on biological activity and on the metabolism of vitamin A acid, show clearly that the compound is very labile in the chick. This property undoubtedly accounts for the decreased biological activity compared with vitamin A alcohol in tests where daily oral supplements are given. Even with a relatively high dose of 100 μg there was an insufficient supply to all tissues so that both eye lesions and transient neurological symptoms appeared. When supplied in the diet so that the chicks received the compound continuously day and night, no signs of vitamin A deficiency developed during the 6-week period. Longer feeding periods would probably be required to determine whether abnormalities would appear similar to those reported in the retina of the rat (Dowling and Wald, '58) or in the reproductive system (Thompson et al., '61a, b).

The sparing of vitamin A in the body by supplements of vitamin A acid could be interpreted as evidence that these two compounds share the same catabolic pathway. A similar sparing effect has recently been reported in the pig.[8]

The tissue fractionation studies showed that vitamin A acid exists both free and protein-bound. In addition, at least two degradation products or metabolites were shown to be formed rapidly. The inability of the fraction C metabolite to give color with antimony trichloride indicates a loss of the conjugated system in the vitamin A acid molecule. The labeling was such that complete oxidation of the side chain would still yield a radioactive metabolite. Garbers et al. ('60) found similar water-soluble products in rats after giving labeled vitamin A alcohol. We have observed that when C^{14} vitamin A acid dispersed in water is shaken at 37°C in air with hemoglobin as a catalyst, a considerable fraction of the activity becomes dialyzable within a few minutes. This shows that the catalyzed oxidation quickly degrades the molecule. In view of the rapid appearance of the water-soluble metabolite in the intestine (within one hour) and also the liver after vitamin A acid is ingested, it is possible that nonenzymatic oxidation is responsible for the fast disappearance of the compound. We have observed (unpublished results) that when C^{14} vitamin A acid was fed to rats, within half an hour the molecule in the intestinal contents had undergone modification. Analysis of the stomach contents at this time showed both unchanged vitamin A acid and metabolities to be present. The alcohol-ether soluble fraction of the intestinal contents gave an instantaneous transient blue color with antimony trichloride, which lasted a fraction of a second and changed within two seconds to gray-blue and finally became colorless. Ultraviolet absorption in absolute ethanol indicated a maximum absorption at 310 mμ. This compound probably is closely related to the first oxidation product of vitamin A in air, reported by Moore ('57). Rats apparently have a more efficient oxidative or degradative system than the chick so that little, if any, unchanged vitamin A acid is admitted into the tissues of the rat.

The recent demonstrations by Futterman ('62) and Elder and Topper ('62) that there are enzyme systems capable of oxidizing vitamin A aldehyde to vitamin A acid, together with the observation of Dmitrovskij ('61) that vitamin A acid is found *in vivo* after administration of retinene to rats, would lend support to the suggestion that vitamin A acid is an important link in the metabolic pathway of vitamin A. However, retinene given to rats orally or by injection, results in the formation and deposition of vitamin A in the tissues, with no trace of the aldehyde being present (Glover et al., '48). By using radioactive retinene, Zachman and Olson

[8] Nelson, E. C., Dehority, B. A., Teague, H. S., Grifo, A. P., Jr. and Sanger, V. L. 1962 The effect of vitamon A acid on cerebrospinal fluid pressure (CSFP) and blood and liver vitamin A concentrations in the pig. Federation Proc., *21*: 474 (abstract).

('61) showed that after intracardial injection the activity in liver was almost completely accounted for by vitamin A esters or alcohol.

In limited experiments (not reported) we were unable to find any evidence for the presence of vitamin A acid in the intestinal contents or mucosa, blood and liver 2, 6 and 18 hours after giving 3 mg of vitamin A aldehyde to depleted chicks. The tissues were homogenized and extracted with alcohol-ether and the extract chromatographed on paper as described above. This negative evidence for the *in vivo* reaction: vitamin A aldehyde → vitamin A acid, is in opposition to the report of Dmitrovskij ('61). Although this reaction has been catalyzed *in vitro* by both an aldehyde oxidase and a dehydrogenase (Futterman, '62; Elder and Topper, '62) the quantitative significance of the aldehyde oxidation in the living animal remains to be demonstrated.

SUMMARY

The amount of vitamin A acid required to prevent vitamin A deficiency symptoms in chicks depended upon the time dosing began relative to the onset of symptoms. The acid was found to have a sparing action on vitamin A in the body. When vitamin A acid was the only source of the vitamin in the diet, chicks grew and developed normally during a 6-week experimental period. When the acid was given in daily oral doses, amounts up to 100 μg would not completely prevent development of ocular or central nervous system lesions, although growth was normal.

When a large oral dose was given, the unchanged vitamin A acid could be detected in the tissues up to 18 hours, but not after this time. No other form of vitamin A was detected. The acid was found to be concentrated primarily in the microsomal fraction of liver. Experiments with C^{14} vitamin A acid confirmed the rapid metabolism or destruction which begins in the digestive tract. Four radioactive fractions, based on solubility, were prepared from chicken liver after a dose of C^{14} vitamin A acid. Two of these represented the unchanged acid while a third fraction, fat soluble, gave no reaction with antimony trichloride. A fourth fraction was water soluble. In a rat curative assay, the third fraction had biological activity but fraction 4 did not. No evidence was obtained for the conversion in the body of vitamin A acid to the aldehyde or alcohol.

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Metabolic Uncouplers, Methionine and Oxygen Consumption of Chicks[1,2]

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The ability of methionine to reduce oxygen consumption of chicks both in the presence and absence of iodocasein (Charkey, '59; Baldini, '61) implies that methionine may be the antithyrotoxic factor. This paper is a speculation on that probability, viewing the studies of this laboratory in the light of other pertinent publications.

Overby et al. ('59) cite one instance (Boldt et al., '58) in which methionine increased survival time of hyperthyroid rats, and two (O'Dell et al., '55; Ershoff, '47) in which it appeared to be inactive. Although O'Dell et al. found no added growth when a mixture of glycine (1%), DL-methionine (0.5%) and L-arginine (0.5%) was added to the basal ration, they felt that the active factor might be an amino acid or combination of amino acids.

O'Dell et al. ('55) using rats under iodocasein stress and treatment with antithyrotoxic factor preparations, found a correlation between growth and intestinal xanthine oxidase activity. This suggested to them that the antithyrotoxic factor may be required to give maximal xanthine oxidase activity when the animal is under stress. In weanling pigs Shirley et al. ('60) noted that liver xanthine oxidase activity increased with higher protein intakes. Williams et al. ('49) provided evidence that rat liver xanthine oxidase activity is abolished in methionine deficiency. There was no effect on endogenous O_2 uptake of liver *in vitro*. Williams et al. ('53) showed that in the rat vitamin B_{12} deficiency also led to reduced xanthine oxidase activity. They considered the effect of vitamin B_{12} to be an indirect one concerned with general protein metabolism, in contrast with the effect on betaine-homocysteine transmethylase, for which vitamin B_{12} was regarded as a cofactor or coenzyme precursor. Mangiantini ('49) concluded that although the thyroid hormone may in part regulate transmethylation, it does not regulate methionine synthesis in rats. Yet Harper et al. ('46) reported that dietary iodocasein or desiccated thyroid, or subcutaneous thyroxine, caused a decrease in rat liver betaine-homocysteine transmethylase activity. Supplementation with dried, defatted pork partially counteracted the effect of iodocasein. Changes in betaine-homocysteine transmethylase activity were quite closely paralleled by changes in growth obtained. Ericson and Harper ('56) indicated that adequate protein, and also methionine and certain other amino acids in excess led to increased levels of rat liver betaine-homocysteine transmethylase. The associated paper of Ericson et al. ('56) indicating a positive role of vitamin B_{12} in rat liver betaine-homocysteine transmethylase activity and in methionine synthesis is also of interest. Vaisler et al. ('60) reported that a daily dose of 20 μg of vitamin B_{12} decreased the oxygen consumption of rats. That methionine does so in chicks appears now to be firmly established.

Thus both methionine and vitamin B_{12} have been reported to exert effects opposite from those of thyroactive materials on certain enzyme systems, and on oxygen consumption *in vivo*. The observed effects of vitamin B_{12}, paralleling to a degree those of methionine, presumably can be accounted for partly at least by its known

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roles in biosynthesis and transfer of methyl groups.

In view of these relationships, and notwithstanding that the diets used were generally well provided with methionine according to accepted standards, the total situation suggests to the present writers that *available* methionine in the various antithyrotoxic supplements used has had much to do with the responses obtained.

Pertinent in this respect is the observation of Rosenberg et al. ('55) that free DL-methionine was of benefit when added to chick starter diets containing ample bound methionine according to accepted standards. The added free methionine still produced statistically significant improvement in body weight gain after such a diet had been further supplemented with fish meal to levels at and above those producing maximal response to fish meal. The authors state, "The statistically significant improvements due to free methionine must then be due to some other effect (than increase in bound methionine via increased fish meal) associated with the presence of fish meal in the diet."

A later publication from the same laboratory in cooperation with others (Rosenberg et al., '55) has definitely established that in chicks a synergistic action occurs between supplemental methionine and fat. In a more recent paper (Baldini, '61), the author concluded that "the ability of a bird to utilize for productive purposes the metabolized calories of a diet is impaired by a methionine deficiency. The metabolized calories that are not put to productive use are given off as heat as evidenced by oxygen consumption studies."

Overby et al. ('59), acknowledging "persistent failure in clear-cut fractionation of an active (antithyrotoxic) principle from liver residue," considered the possibility of nonspecific response from cruder materials, and sought the explanation in terms of amino acid imbalance. Their review of the pertinent literature has already been cited. In the studies of the present writers' laboratory, the diet was balanced with respect to all essential amino acids except for the methionine-cysteine deficiency, and for histidine which was present at three times the National Research Council ('54) requirement level for a 20% protein diet.

Such a level of histidine may have conditioned the responses obtained, in view of the observation of Brown et al. ('60) that methionine influences histidine metabolism. In rats normal with respect to vitamin B_{12} and folic acid supply, methionine consistently depressed CO_2 production from the number 2 carbon of histidine. Homocysteine had no such effect.

In sum the situation stands thus: The most consistently antithyrotoxic substances, according to Overby et al. ('59) have been liver residue and unsaturated fats. The role of methionine has been in doubt because of conflicting results obtained. It cannot be assumed that all of the methionine in a mixed diet is of equal availability. All of the substances reported to have antithyrotoxic activity might be expected to contain bound methionine, as well as vitamin B_{12}, with the exception of unsaturated fats. Dietary fat has much to do with methionine — vitamin B_{12} interrelationships, and with the effects of methionine in energy metabolism. Vitamin B_{12} probably exerts its effects by methyl synthesis or transfer reactions enabling more efficient synthesis of methionine or of some metabolite which spares methionine. In studies in this laboratory using oxygen consumption *in vivo* as an additional criterion of thyroid function, crystalline DL-methionine has counteracted all effects of iodocasein in a highly consistent manner.

The possibility that methionine is, after all, the main ingredient of the elusive antithyrotoxic factor requires further investigation.

Although 2,4-dinitrophenol (DNP) is not thyroactive, its established role as a metabolic uncoupler of oxidative phosphorylation aroused interest in the question of whether methionine would reverse the effects of DNP also. If so, it would be further indicated that the ability of methionine to moderate oxygen consumption is of a rather general character, and not to be considered a specific antagonistic action toward the thyroid hormone. If not, the hypothesis that methionine has an antithyrotoxic action would be made more tenable. With the foregoing in mind, several experiments have now been carried

out with chicks, involving DNP as a metabolic uncoupler *in vivo*.

Although DNP has been widely used in biochemical investigations *in vitro* as a metabolic uncoupler, there is a dearth of information regarding its administration and action in chicks. Literature reports of its use in a variety of biological systems, including certain laboratory animals, permitted a rough estimate of 5 to 10 mg DNP/kg body weight as the lethal dose by injection. As to dietary administration, particularly to chicks, there was little guidance available.

Accordingly a preliminary experiment, not reported here in detail, was run to ascertain physiologically effective, tolerated levels of administration, both orally and by injection. The results of this experiment indicated 50 mg of DNP/kg of diet as a suitable level of dietary administration. This level was given in experiment 40, to be described. As to injected DNP, chicks weighing 200 to 250 gm tolerated with no overt effects 10 μg and 100 μg of DNP injected in saline into breast muscle. A dose of 1000 μg was lethal in most cases within a few hours, indicating that in this particular trial 5 mg DNP/kg body weight was lethal.

PROCEDURE

The animals used were one-day-old Single Comb White Leghorn cockerels from a local hatchery. Experimental groups were selected after a two-day stabilizing period when they were fed corn meal, by a systematic weight equalization procedure, and placed in individually heated, thermostatically controlled, wire-mesh bottom, all-metal pens in batteries housed in an air conditioned, constant temperature animal room. Twenty-six chicks per group were started in experiment 39, and 27 per group in experiment 40. In experiment 40, to avoid crowding and to control cannibalism which occurred in one group, the numbers were reduced to 20/group after one week and again to 10/group after three more weeks. Eliminations were made by the same rigid rule in all pens, removing the weight extremes in each case.

The chicks were fed and watered ad libitum. The basal diet for experiment 39 consisted, in per cent, of yellow corn meal, 66.0; soybean meal (50% protein), 13.5; dehydrated alfalfa meal (17% protein), 5.88; dried brewer's yeast, 5.00; gelatin, 4.00; steamed bone meal, 1.70; limestone, 1.00; DL-phenylalanine, 0.214; DL-tryptophan, 0.041; iodized salt, 0.500; potassium chloride, 0.200; magnesium sulfate, 0.242; and vitamins and trace minerals in milligrams per kilogram of diet as follows: $MnSO_4$, 50; $FeSO_4$, 20; $CuSO_4$, 2.0; $ZnCl_2$, 0.2; $CoCl_2$, 0.2; pyridoxine·HCl, 2.50; folic acid, 0.50; biotin, 0.10. For experiment 40, this same diet plus 4.0 kg of corn oil/100 kg of the diet was used. This change was made to eliminate a certain amount of dustiness and stickiness from gelatin.[3] Vitamin B_{12} was plentifully supplied (25 μg/kg diet) by the basal diets, in order to assure clear-cut responses to methionine.

In experiment 39, DNP was administered by injection in olive oil solution under the loose skin of the neck. In control groups the same amount of the same oil was injected on the same schedule. During the course of the 5-week feeding trial there was a gradual increase in dose of DNP, not only to adjust to weight gains, but also per unit of body weight. This was such that by the end of the feeding trial the birds were receiving 15 mg DNP/kg body weight, instead of the initial 5 mg/kg. The increases were kept parallel in DNP groups, as were increases in olive oil volume in all groups. The volume of oil varied from an initial 0.20 ml/bird to 0.40 ml. The dosage was increased since there were no overt effects or growth depression from the DNP. All measurements of O_2 uptake were made within an interval 2 to 6 hours after injection.

In experiment 40, the DNP was administered in the diet at a level of 50 mg/kg of diet.

Body weight, feed consumption and oxygen consumption with the apparatus of Charkey and Thornton ('59) and, in experiment 40, hemoglobin, were determined. No anemia was observed in ex-

[3] We are indebted to the Dow Chemical Company, Midland, Michigan, for synthetic amino acids used in balancing diets.

periment 40 nor were there any gross abnormalities of the organs in either experiment.

RESULTS

A. With injected DNP (experiment 39). Methionine produced definite increases in growth over unsupplemented groups, both in chicks injected with DNP and in uninjected birds (table 1). The DNP appeared to reduce growth slightly in the presence of added methionine but not in methionine deficiency. In respect to feed utilization efficiency, DNP likewise had less effect in the methionine-deficient birds than in those fed the balanced diet. This is a better criterion for the purpose at hand than growth as such, and it indicates that DNP reduced efficiency more readily when efficiency was high than when it was already reduced somewhat by a methionine deficiency.

Oxygen uptake measurements were made throughout the feeding trial and for several weeks beyond, to provide a more extended series of observations. At termination of the recorded feeding trial the 6 birds weighing closest to the average for each pen continued to receive the same diets and injections (15 mg DNP/kg body weight) and were rotated in O_2 uptake measurements. All data on O_2 uptake for the first 45 days of experiment 39 are summarized in table 2 as averages for each of 6 age periods established in summarizing the data. It appears that DNP increased oxygen consumption of intact chicks when measured within 6 hours following its injection. This effect was highly significant ($F = 42.41$; $F_{0.01} = 6.81$). Methionine appeared to have no ability whatever to counteract the effect of DNP, under the indicated conditions of measurement. In the absence of DNP, methionine may have reduced oxygen consumption slightly — an effect found in earlier experiments and reported to be highly significant (Charkey, '59; Baldini, '61). Because the results in the present experiment were not clearcut at 45 days of age, the series of observations was continued in the absence of DNP to 72 days of age. The additional observations continued the trend which had begun to appear after

TABLE 1

Growth and feed consumption (experiment 39)

	Average body weight						Feed utilization efficiency				
Weeks on experiment	0	1	2	3	4	5	1st	2nd	3rd	4th	5th
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	%	%	%	%	%
Basal	38[1](26)	67(26)	117(26)	179(26)	255 ± 5(26)	340 ± 7(26)	47.1	44.4	38.2	35.2	32.6
+ Methionine[2]	38 (26)	68(25)	121(25)	197(25)	277 ± 7(25)	376 ± 9(25)	43.7	50.3	50.1	37.4	36.5
+ DNP[3]	38 (26)	66(26)	114(26)	183(26)	256 ± 7.5(26)	336 ± 10(24)	46.7	45.9	41.6	34.0	31.9
+ Methionine[2] + DNP[3]	38 (26)	66(26)	117(26)	191(26)	263 ± 5(24)	362 ± 7(24)	45.5	48.0	47.0	32.3(?)	36.8

[1] Mean ± SE of mean for number of birds shown in parentheses.
[2] 0.36% DL-methionine in diet.
[3] Injected, see text.

TABLE 2

Average oxygen uptake of chicks in vivo (experiment 39)

Age in days	Basal	+ Methionine[1]	+ DNP[2]	+ Methionine[1] + DNP[2]
	ml dry O_2/kg chick/sec[3]	*ml dry O_2/kg chick/sec*[3]	*ml dry O_2/kg chick/sec*[3]	*ml dry O_2/kg chick/sec*[3]
6–9	0.441(3)[4]	0.414(4)	0.400(3)	0.400(4)
13–17	0.449(9)	0.475(8)	0.483(9)	0.487(8)
20–24	0.405(7)	0.404(10)	0.473(7)	0.456(10)
27–30	0.417(6)	0.389(6)	0.481(6)	0.509(6)
35–38	0.381(5)	0.362(5)	0.501(6)	0.506(6)
41–45	0.372(7)	0.332(7)	0.410(6)	0.407(6)
Avg	0.411	0.396	0.458	0.461

[1] 0.36% DL-methionine in diet.
[2] Injected; see text.
[3] Corrected to standard conditions.
[4] Figures in parentheses indicate the number of loadings of the O_2 uptake apparatus for each average.

TABLE 3

Growth and feed consumption (experiment 40)

Treatment	Avg body weight[1]			Avg feed utilization efficiency	
	2 Weeks	4 Weeks	6 Weeks	0–4 Weeks	0–6 Weeks
	gm	*gm*	*gm*	%	%
Basal diet	109 ± 1.6[2](20)	253 ± 4(20)	411 ± 3(10)	46.9	40.5
Basal diet (duplicate)	109 ± 1.7 (20)	249 ± 5(20)	396 ± 5(10)	44.2	38.2
+ 0.2% DL-methionine	123 ± 1.9 (20)	289 ± 6(20)	485 ± 5(10)	52.7	46.9
+ 0.6% DL-methionine	117 ± 1.8 (20)	287 ± 5(20)	502 ± 8(10)	56.5	49.5
+ 0.05% iodocasein	95.0 ± 2.1 (20)	230 ± 7(20)	391 ± 5(10)	45.2	39.5
+ 0.2% DL-methionine + 0.05% iodocasein	110 ± 2.0 (20)	285 ± 5(20)	493 ± 5(10)	53.2	44.4
+ 0.6% DL-methionine + 0.05% iodocasein	107 ± 2.6 (19)	276 ± 8(19)	490 ± 9(10)	48.3	41.9
+ 50 ppm 2,4-dinitrophenol	109 ± 2.2 (20)	237 ± 5(20)	376 ± 5(10)	43.3	39.3
+ 0.2% DL-methionine + 50 ppm 2,4-dinitrophenol	122 ± 2.2 (20)	288 ± 7(20)	495 ± 5(10)	52.3	46.8
+ 0.6% DL-methionine + 50 ppm 2,4-dinitrophenol	121 ± 2.4 (20)	294 ± 6(20)	495 ± 5(10)	48.6	43.9

[1] Average body weight in all groups was 36.5 ± 0.5 gm at start of experiment.
[2] Mean ± SE of mean for number of birds shown in parentheses.

24 days of age (see table 2), and analysis of the entire extended series again disclosed a highly significant effect of methionine ($F - 21.32$; $F_{0.01} - 7.39$).

Also oxygen consumption decreased with maturation of the chicks, in the absence of DNP (table 2). This effect has been documented previously (Sturkie, '54). The DNP prevented this normal drop in oxygen consumption up to about 5 weeks of age. But again, it must be borne in mind that in this experiment all measurements were made within 6 hours following injection. It cannot be concluded that compensatory reductions did not occur in the intervals between injections.

B. With dietary administration of DNP (experiment 40). This experiment provided an opportunity to observe the effects of DNP under constant dietary administration, and to compare these effects with those of iodocasein given in the same way in the same experiment. The 6-week feeding trial data are summarized in table 3.

TABLE 4

Growth effects of iodocasein and dinitrophenol (DNP) in presence and absence of methionine (experiment 40)[1]

Age of chicks	Effects of iodocasein		Effects of DNP	
	Methionine-deficient	Methionine-supplemented	Methionine-deficient	Methionine-supplemented
weeks	*gm*	*gm*	*gm*	*gm*
1	−6.0	−4.5	2.5	0.5
2	−14.0	−11.5	0.0	1.5
3	−21.5	−12.5	−3.5	1.0
4	−21.0	−7.5	−14.0	3.0
5	−9.0	−3.0	−27.0	−1.5
6	−12.5	−2.0	−27.5	1.5
Avg	−14.0	−6.8	−11.6	1.0

[1] Minus sign indicates growth depression from that of indicated control groups. Figures shown in "methionine-supplemented" columns are averages of effects at both levels of methionine supplementation (calculated from the values, 0.2 and 0.6% DL-methionine, table 3), since these did not differ materially.

The picture obtained was one of greatly improved growth and feed utilization efficiency as a result of methionine supplementation. Both DNP and iodocasein appeared to reduce growth somewhat below that obtained in the methionine-deficient basal groups. The growth depression from iodocasein was reduced in the presence of adequate methionine; but that from DNP was completely obliterated by the methionine (table 4).

Oxygen uptake *in vivo* was measured in all groups throughout the course of the feeding trial; and for several weeks thereafter, on two birds nearest average weight for each group and maintained with the same diets. To avoid possible diurnal effects, pen orders for O_2 uptake measurement were randomized for each cycle of observations throughout the experiment. The results are summarized in table 5. Although iodocasein consistently elevated oxygen consumption by the chicks, DNP did not. Reductions obtained by methionine supplementation were of the same order of size in the presence or absence of DNP, but much greater in the presence of iodocasein than in its absence.

DISCUSSION

Dietary iodocasein caused consistent increases in oxygen consumption *in vivo* by chicks. The ability of DNP to do so, when administered in the same way, is in doubt. The present studies *in vivo* lend further support, then, to the notion (Judah, '51; Tapley, '56; Shaw et al., '59) derived from studies *in vitro* that the metabolic action of DNP differs in some way from that of thyroxin. Injected DNP induced short-term increases in oxygen consumption, with no apparent effect on growth or feed utilization efficiency. These effects on oxygen consumption, unlike those from fed iodocasein or from methionine deficiency, were not prevented by supplemental methionine. Dietary iodocasein and dietary DNP had similar effects on growth and feed utilization efficiency. Both reduced growth somewhat from that obtained in methionine-deficient basal groups. In the presence of supplemental methionine at nutritionally adequate to superabundant levels, iodocasein still produced slight growth depression, but the ability of DNP to do so was completely abolished. Here then is evidence that the relationship of methionine to the two stressors, one thyroactive and the other not, is fundamentally different. It is beyond the scope of this paper to speculate on the nature of the difference. But the apparent existence of a difference gives more credence to the idea that methionine may indeed possess some rather specific ability as an antithyrotoxic compound. The ability of methionine to prevent growth depression by DNP appears to rest on some other property or function of methionine.

Further studies should be conducted to ascertain (a) whether and how generally other amino acid deficiencies have ramifications in oxidative metabolism similar to those of methionine deficiency; (b) whether other metabolic uncouplers or 2,4-dinitrophenol in higher dietary dosage,

TABLE 5

Average oxygen uptake of chicks in vivo as percentage of basal (experiment 40)

	Age in days																		Avg
	14	17	21	24	28	31	35	38	42	45	49	52	56	59	63	63	70	73	
Avg of 2 basals (ml O_2/kg/sec)	0.508	0.510	0.520	0.453	0.478	0.434	0.437	0.416	0.350	0.333	0.357	0.281	0.261	0.215	0.225	0.207	0.229	0.201	
Avg of 2 basals (ml O_2/kg/sec) (adjusted)[1]	0.520	0.510	0.500	0.490	0.466	0.448	0.420	0.400	0.367	0.343	0.307	0.280	0.255	0.240	0.225	0.217	0.205	0.200	
0.2% Methionine	96	96	90	90	86	86	86	119	98	95	100	91	88	94	101	95	89	99	94
0.6% Methionine	88	94	97	95	93	92	89	69	91	83	94	91	83	85	84	83	88	92	88
0.05% Iodocasein	105	107	110	122	89	107	115	116	117	124	126	101	118	107	109	103	112	116	111
Iodocasein + 0.2% methionine	91	91	102	98	99	86	95	102	105	109	102	82	88	95	89	111	99	96	97
Iodocasein + 0.6% methionine	—	—	—	94	91	91	93	123	113	100	95	96	88	87	93	90	99	94	96
DNP, 50 ppm	95	111	98	108	89	105	115	82	111	108	111	109	82	101	76	97	109	96	100
DNP + 0.2% methionine	91	99	96	95	93	94	94	97	75	108	87	85	101	96	99	89	93	106	94
DNP + 0.6% methionine	91	94	81	95	92	85	97	122	88	89	96	84	90	84	82	84	81	83	90

[1] Figures shown in this line are the results of a graphical "curve-smoothing" procedure applied to the actual figures obtained in the basal group (shown in 1st line). Percentages shown are referable to the adjusted basal figures in line 2.

can be seen to resemble thyroactive substances more closely in their action; and (c) whether injected thyroxin exerts a short-lived action, insensitive like that of injected DNP to the level of methionine in the diet.

SUMMARY

After estimation of effective tolerated dosages of 2,4-dinitrophenol (DNP) in preliminary trials, this substance has been studied as a metabolic stressor in chicks. The DNP was administered by injection in one study, orally in the diet ad libitum in the other. In the latter study, DNP was compared directly to iodocasein, in the presence and absence of supplemental methionine, to observe similarities and dissimilarities in interactions of the two with methionine.

Iodocasein and DNP were observed to differ in their effects on oxygen consumption *in vivo*, but to cause similar effects on growth in methionine deficiency. Methionine showed different preventive actions toward iodocasein and DNP.

The pertinent literature was reviewed. In that context the presently reported observations have been interpreted as strongly indicative of an antithyrotoxic action of methionine, conditioned by a combination of other dietary factors.

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Effect of Dietary Orotic Acid on Liver Proteins[1]

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The present work is the continuation of a study on the effects of dietary orotic acid on the livers of male albino rats. As reported by Standerfer and Handler ('55), orotic acid in the diet gives rise to fatty infiltration of the liver. More detailed studies (Handschumacher et al., '60) have shown that 1% of orotic acid in the diet brings about rapid fatty infiltration of the liver, and that this can be nullified or prevented by the addition to the diet of 0.25% of adenine sulfate. This type of fatty liver does not appear to be related to that caused by a choline-deficient diet. Changes in pyrimidine metabolism were noted, and it was observed further that the amount of protein in the liver of such rats is altered by the ingestion of orotic acid. The results of investigations of the type of fat which accumulates in the livers of treated animals have been detailed by Creasey et al., '61). The present study is an attempt to elucidate the effect of orotic acid on the proteins of the liver.

EXPERIMENTAL

Male albino rats of the Osborne-Mendel strain were weaned at 20 to 22 days and placed directly on test. The basic purified diet contained: (in percent) vitamin-free casein,[2] 18; sucrose, 72.8; corn oil,[3] 2; salts,[4] 5.0; and vitamin mixture,[5] 2.2. Additional choline chloride was added at the rate of 1.35 gm/kg of diet. Orotic acid was added at the expense of all other ingredients. When used, adenine sulfate was added at the rate of 0.25% to the diet and fed during the entire test period. Care of the animals has been described previously (Handschumacher et al., '60). The livers were frozen immediately after removal from the animals.

Fat determinations were made with a Nolan extraction apparatus[6] (Nolan, '49). About 0.5 to 1.0 gm of liver was weighed into a tared alundum crucible (porosity, coarse, RA98) and mascerated in a little 95% ethanol against the side of the crucible with a glass rod. Material adhering to the rod was washed off with additional ethanol. The liver was extracted for 5 hours with 95% ethanol, and subsequently for 5 hours with ether (purified for fat extraction). After being dried, the residue was weighed and the amount of fat calculated by difference, a moisture determination previously having been made on a portion of the original tissue.

Protein was determined in the defatted residue by a micro-Kjeldahl technique for digestion followed by the use of Nessler's reagent. The soluble protein fraction was prepared, in the cold, by grinding a portion of the frozen liver in a Potter-Elvehjem homogenizer with 0.02 M phosphate buffer, pH 7.0. The suspension thus obtained was centrifuged three times at 12,000 $\times$ *g* in a refrigerated centrifuge, the sediment and fat being discarded each time. The supernatent was dialyzed for 21 hours at 2 to 3°C against the phosphate buffer.

Lipoproteins were isolated from the tissue by the calcium oxalate precipitation method of Sandor et al. ('57). The amount of fat in the lipoprotein fraction was determined by first treating the material with hot 95% ethanol followed by extraction of

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[1] This work was supported by a grant from the Nutrition Foundation Inc. (no. 321).
[2] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.
[3] Mazola, Corn Products Company, Argo, Illinois.
[4] Salt mixture (Hubbell et al., '37) obtained from Nutritional Biochemicals Corporation.
[5] Obtained from Nutritional Biochemicals Corporation, Cleveland, and added at a rate to provide the following amounts per 100 gm of diet: vitamin A, 1980 units; vitamin D, 220 units; α-tocopherol, 11 mg; ascorbic acid, 99 mg; inositol, 11 mg; choline chloride, 165 mg; menadione, 4.95 mg; *p*-aminobenzoic acid, 11 mg; niacin, 9.9 mg; riboflavin, 2.2 mg; pyridoxine·HCl, 2.2 mg; thiamine·HCl, 2.2 mg; Ca pantothenate, 6.6 mg; biotin, 44 μg; folic acid, 198 μg; and vitamin B_{12}, 2.97 μg.
[6] Macalaster Bicknell Company, New Haven, Connecticut.

the fat with ether. Ribonucleic acid was determined by the method of Schneider ('57).

RESULTS

The appearance of fat in the livers of animals fed a diet containing 1% of orotic acid was first evident on the third to fourth day of test. By the fourth day the increase over the controls was about 3% of fat, and increased to about 23% by the tenth day. Changes in total protein per liver were more subtle, but were observable on the eighth day of test. The level of protein was increased in animals receiving orotic acid. When calculated as the percentage of protein, the change was also evident on the eighth day (table 1). By the tenth day the change in total protein was quite marked and, if the animals were kept 20 days, the amount of protein per liver in the treated animals was almost doubled.

Since total protein is at best only a partial indication of liver activity, the soluble protein fraction of the liver was prepared to determine whether the changes noted in the total protein applied to the soluble fraction as well. The results are shown in table 2 and are calculated on the fat-free basis to preclude misleading differences arising from the variability of the fat content. The amount of protein in the soluble fraction in the livers of the animals receiving orotic acid increased. The inclusion of adenine in the diet nullified this protein build-up. The total solids in the soluble fraction of the livers from animals fed both orotic acid and adenine is as high as those fed adenine alone, but the amount of protein did not increase correspondingly. The nature of the excess solids is not known at this time. It appears that adenine not only prevents the increase of liver fat, but also prevents an

TABLE 1

Changes in fat and protein in livers of rats fed 1% of orotic acid

Days on test and treatment[1]		Liver wt	Fat	Protein	
		gm	%	*gm/liver*	%
0	(9)[2]	1.99	7.28	0.33	19.4 ± 1.4[3]
2–C	(3)	2.24	6.71	0.32	14.2 ± 0.2
2–OA	(3)	2.88	6.15	0.39	13.5 ± 0.2
4–C	(9)	2.56	7.36	0.45	16.7 ± 0.8
4–OA	(13)	2.64	10.55	0.48	17.5 ± 1.0
6–C	(7)	3.12	9.01	0.52	16.3 ± 0.9
6–OA	(8)	3.88	19.27	0.50	14.5 ± 1.0
8–C	(8)	4.34	8.60	0.73	16.7 ± 0.7
8–OA	(9)	5.64	27.31	0.79	14.1 ± 0.3
10–C	(15)	4.79	7.94	0.82	16.3 ± 0.4
10–OA	(13)	7.21	31.68	0.92	12.4 ± 0.8
12–C	(2)	4.16	5.45	0.74	17.9
12–OA	(2)	9.48	33.02	0.95	10.1
20–C	(2)	5.51	6.83	0.89	16.2
20–OA	(2)	14.45	34.83	1.57	10.9

[1] The designations C and OA refer to control and orotic acid.
[2] Numbers in parentheses indicate number of animals.
[3] SE indicated where applicable.

TABLE 2

Protein and total solids of the soluble fraction of livers of rats fed test diets for 10 days

Dietary treatment	Total solids	Protein
	mg/gm fat-free liver	
Control (4)[1]	93.8	81.0
1% Orotic acid (4)	118.9	104.7
1% Orotic acid + 0.25% adenine sulfate (4)	124.0	72.3
Control + 0.25% adenine sulfate (4)	81.4	74.7

[1] Numbers in parentheses indicate number of animals.

increase in protein. There are reports that lipids play a role in the final stages of protein synthesis (Hunter and Godson, '62), but there is no assurance that this applies in the present case.

An investigation of lipoproteins in the liver was made since it was reasonable to expect that these components would be increased in the livers of animals ingesting orotic acid. However, the amount of liver lipoprotein did not increase in animals fed orotic acid, but rather decreased (table 3). Nevertheless the data suggest that the type of lipoprotein in livers from treated animals is different from that in the controls, since the amount of fat in the liver lipoproteins from treated rats is more than twice that found in those from the livers of control rats.

It has already been shown that RNA levels in the livers of rats fed orotic acid are higher than those in livers of control rats (Handschumacher et al., '60). However, it was advisable to examine the RNA content of the soluble protein fraction, for such information might give an indication of the amount of nucleoprotein present. These data are summarized in table 4. Although the results do not indicate a large difference in RNA values, they do not preclude the possibility that the nucleoproteins are in fact different. An increase was noted in animals treated with orotic acid and adenine in agreement with the data of Handschumacher et al. ('60).

The use of an animal fat (butter), instead of a vegetable fat in the basal diet, or the deletion of all fat, had no effect on fat accumulation in the livers of rats fed a diet containing orotic acid.

DISCUSSION

Although the amount of lipoprotein in the liver of treated animals diminished, the proportion of fat in these lipoproteins was higher than in the controls. Since lipoproteins are involved in fat transport, it was expected that the lipoprotein fraction should have increased in the livers of the treated animals to help compensate for the excess fat accumulation. This was not the case, and further considerations are therefore necessary. The amount of fat in the lipoproteins from treated livers increased, but may represent only fat accumulation of the type already present. If an actual change in the lipid composition has occurred, then a different biochemical pathway is involved. This matter must wait until the lipids of the particular lipoprotein fractions are investigated.

Large amounts of fat in the liver brought about by ingestion of orotic acid may be due to rapid mobilization of depot fat. Whether this holds true for the increased amount of fat in the lipoproteins is ques-

TABLE 3

Lipoprotein values in livers of rats fed test diets for 10 days

Dietary treatment	Lipoprotein	Fat in lipoprotein	
	mg/gm solids	*mg/gm solids*	*%*
Control (5)[1]	104.5	10.5	10.0
1% Orotic acid (4)	88.5	20.9	23.6
1% Orotic acid + 0.25% adenine sulfate (4)	107.7	10.6	9.8

[1] Numbers in parentheses indicate number of animals.

TABLE 4

Ribonucleic acid (RNA) in soluble protein fraction from livers of rats fed 1% of orotic acid for 10 days

Dietary treatment	RNA/gm protein	RNA as % of protein
	mg	
Control (4)[1]	30.85	8.04
1% Orotic acid (4)	21.99	7.07
1% Orotic acid + 0.25% adenine sulfate (4)	20.89	12.25

[1] Numbers in parentheses indicate number of animals.

tionable, since adenine prevents accumulation both in total liver fat and in the lipoprotein fraction. Possibly the build-up of lipoprotein fat is a mechanism designed to rid the liver of excess fat to the circulation as the stable, but soluble, lipoprotein complex. It has been reported that rats injected with puromycin evidenced decreased hepatic lipoprotein synthesis associated with fatty livers (Robinson and Seakins, '62). These results are similar to the present observations.

An imbalance of amino acids in the diet can also lead to fatty livers. Fat increase caused by threonine deficiency can be modified by caloric restriction (Yoshida et al., '61). Fatty livers induced by choline deficiency do not react in this manner. In the present work, a decrease of calories was accomplished by the substitution of 58.8% of the sucrose with cellulose in the diet. Animals fed such a diet supplemented with 1% of orotic acid for 10 days still accumulated large amounts of fat in the liver, thus suggesting that the fatty infiltration is probably not due to excess caloric intake. It was noted that animals fed either the control or test diets always consumed about the same amounts of food, and the gains in weight were similar. The possibility exists that inclusion of orotic acid in the diet brings about an amino acid imbalance by causing one or more of the amino acids to be limiting, or it may bring about a need for additional amounts of a particular amino acid. In any event, this cannot explain fully the reversal by adenine of fatty infiltration induced by orotic acid.

SUMMARY

The ingestion by albino rats of a diet containing 1% of orotic acid brought about not only fatty infiltration of the liver but also an increase in the total and the soluble protein. Adenine nullified both the fat and protein build-up. An investigation of the lipoprotein fraction showed that there was less in the livers of treated animals, but the percentage of fat in these lipoproteins was higher than that found in the control livers.

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A Comparative Study of the Effects of Bile Acids and Cholesterol on Cholesterol Metabolism in the Mouse, Rat, Hamster and Guinea Pig[1]

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Because of the apparent relationship of blood cholesterol concentrations to atherosclerosis, a large volume of literature has accumulated on the effects of this sterol on tissue cholesterol concentrations and synthesis rates in various species (Kritchevsky, '59; Cook, '59; Portman and Stare, '59). Bile acids, major end products of cholesterol metabolism (Siperstein, '52; Danielsson, '60), also influence the rate of cholesterol turnover (Beher and Baker, '62; Myant and Eder, '61; Seitz and von Brand, '61), and several groups of investigators have studied the effects of these substances on tissue cholesterol metabolism (Howe and Hutchison, '62; Portman and Bruno, '61; Beher and Baker, '61; Sugihara, '61). Unfortunately a great deal of confusion exists as to the relative effects of cholesterol and bile acid dietary supplements, because the investigations have been carried out on various animal species, maintained with different diets.

The present experiment was designed to study the relative influence in the different species of a *standard diet* supplemented in various ways — with cholesterol, corn oil, and several bile acids. Effects on serum, liver and carcass cholesterol concentrations and on synthesis rates were observed in mice, rats, hamsters and guinea pigs. Diets containing chenodeoxycholic and cholic acids were chosen for investigation, because these acids are present as the major components of the bile acid fraction in the bile of the species studied. Hyodeoxycholic and lithocholic acids were of interest, because these substances have been found effective in lowering tissue cholesterol concentrations in mice (Beher et al., '59; Howe et al., '60). An abundant literature exists with respect to cholesterol metabolism in the mouse and rat; fewer studies have appeared on the hamster and guinea pig.

METHODS

The types and strains of animals used were as follows: 100 female albino mice of the Webster strain, weighing 22 to 25 gm; 70 female Sprague-Dawley albino rats, weighing 225 to 250 gm; 70 female Golden hamsters, weighing 90 to 110 gm; and 36 female albino Himalayan guinea pigs, weighing 350 to 400 gm. After a two-week observation period, the mice, rats and hamsters were each divided into 10 equal groups and fed a basal diet (ground commercial rat diet,[2] containing protein, 24.27%; fat, 4.15%; fiber, 4.86%; carbohydrates, 56.23%; and ash 7.78%) supplemented with cholesterol, corn oil, with or without bile acids, in amounts indicated in the tables. Cholesterol and bile acids were dissolved in peroxide-free ether and mixed mechanically with the ground food. The diets containing corn oil were kept in sealed glass containers to prevent deterioration. The guinea pigs were fed only 6 of the diets tested, and each guinea pig received a leaf of lettuce daily. All animals were fed ad libitum, and the various supplements did not alter daily food consumption as determined by weight.

After the animals had been fed the various diets for three weeks, they received intraperitoneal injections of acetate-1-C^{14}. Mice received 5 μc each; rats,

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

40 μc; hamsters, 25 μc; and guinea pigs, 50 μc. One hour after injection, blood samples were taken by heart puncture, following which the animals were decapitated. Livers were removed and quick frozen. Gastrointestinal tract contents were removed and the clean tract combined with the remaining organs and tissues for carcass analysis. Serum cholesterol was determined by the method of Sperry and Webb ('50); liver cholesterol by a previously described method (Beher and Anthony, '58). For carcass cholesterol determination, whole carcasses were homogenized in Waring Blendors of suitable size, and aliquots assayed by the same method as the livers. For determination of cholesterol-C^{14}, cholesterol was isolated as the digitonide, purified by ether-acetone (2:1) and ether washes, plated by the filtration tower technique, and counted in a windowless gas-flow counter.

RESULTS

Effects of the various dietary supplements on serum and liver cholesterol levels are presented in tables 1 and 2, respectively.

Basal diet. Liver cholesterol concentrations were the same in the rat, hamster and guinea pig; and slightly but significantly higher in the mouse. Serum cholesterol concentrations varied greatly from species to species, the highest level and standard deviation being seen in the hamster, the lowest in the guinea pig. These results agree with those reported by Lee et al. ('59).

Corn oil, 3%. Supplementing the diet with corn oil had little or no effect on cholesterol concentrations in serum or liver in any of the species studied.

Cholesterol, 1%. This dietary supplement brought about small but significant increases in serum cholesterol in the mouse ($P = 0.03$) and rat ($P < 0.01$). There were considerable increases in the hamster (6 times) and guinea pig (5 times). Liver cholesterol concentrations increased 3 times in the mouse and rat; 10 times in the guinea pig; and 40 times in the hamster.

Cholesterol, 1% and corn oil, 3%. Mouse and rat serum cholesterol concentrations increased slightly, but to no greater extent than with cholesterol alone. However, in the hamster there was a large increase, which was somewhat greater than with the 1% cholesterol supplement. Liver cholesterol concentrations in the mouse and hamster were similar to those observed when cholesterol was the sole supplement. On the other hand, rat liver cholesterol concentrations increased threefold.

TABLE 1

Effects of diets supplemented with bile acids or cholesterol, or both, on serum cholesterol levels

Basal diet supplements	Mouse	Rat	Hamster	Guinea pig
		mg/100 ml serum		
None	107 ± 5.6[1]	87 ± 11	135 ± 27	50.7 ± 4.8
Corn oil, 3%	114 ± 4.1	80.0 ± 14	138 ± 17	—
Cholesterol, 1%	149 ± 40.3	111 ± 18	633 ± 252	242 ± 28
Cholesterol, 1% + corn oil, 3%	156 ± 16.6	110 ± 15	728 ± 143	—
Cholesterol, 1% + cholic acid, 0.5%	350 ± 63	642 ± 203	858 ± 232	343 ± 49
Cholesterol, 1% + cholic acid, 0.5% + hyodeoxycholic acid, 1%	104 ± 14.2	163 ± 26	542 ± 104	—
Cholic acid, 0.5%	140 ± 9.4	87.8 ± 5.8	234 ± 22	77.6 ± 12.8
Hyodeoxycholic acid, 1%	90 ± 11.5	106 ± 11	165 ± 12	92.5 ± 46
Lithocholic acid, 1%	101 ± 24.8	137 ± 20	252 ± 52	—
Chenodeoxycholic acid, 0.5%	107 ± 9.1	106 ± 18	212 ± 44	74.0 ± 7.4

[1] SD.

TABLE 2

Effects of diets supplemented with bile acids or cholesterol, or both, on liver cholesterol levels

Basal diet supplements	Mouse	Rat	Hamster	Guinea pig
		mg/gm liver[1]		
None	3.10 ± 0.31[2]	2.58 ± 0.29	2.37 ± 0.19	2.61 ± 0.18
Corn oil, 3%	2.96 ± 0.30	2.93 ± 0.25	2.50 ± 0.08	—
Cholesterol, 1%	9.03 ± 2.21	8.38 ± 0.66	83.5 ± 12.7	24.1 ± 3.7
Cholesterol, 1% + corn oil, 3%	12.6 ± 2.15	25.3 ± 6.10	94.2 ± 9.12	—
Cholesterol, 1% + cholic acid, 0.5%	39.9 ± 10.9	56.3 ± 8.9	98.0 ± 7.4	42.4 ± 2.8
Cholesterol, 1% + cholic acid, 0.5% + hyodeoxycholic acid, 1%	3.07 ± 0.52	26.3 ± 7.50	52.0 ± 17.2	—
Cholic acid, 0.5%	5.82 ± 1.77	3.01 ± 0.33	3.38 ± 0.44	4.35 ± 0.60
Hyodeoxycholic acid, 1%	2.74 ± 0.34	2.82 ± 0.16	2.34 ± 0.10	3.40 ± 0.68
Lithocholic acid, 1%	3.23 ± 0.25	2.77 ± 0.22	3.49 ± 0.27	—
Chenodeoxycholic acid, 0.5%	2.64 ± 0.28	2.76 ± 0.29	3.17 ± 0.48	3.80 ± 0.58

[1] Wet weight.
[2] SD.

Cholesterol, 1% and cholic acid, 0.5%. Large increases occurred in serum cholesterol concentrations in all the species. The largest increases were noted in the rat, hamster and guinea pig (6 to 7 times), whereas the mouse exhibited only a threefold increase. The highest absolute serum cholesterol concentration was observed in the hamster. Liver cholesterol concentrations increased greatly in all of the species.

Cholic acid, 0.5%. Cholic acid caused small but significant increases in serum cholesterol concentrations in the mouse ($P < 0.01$) and guinea pig ($P < 0.01$). No effect was noted in the rat. In the hamster, serum cholesterol concentrations were doubled.

There were small but significant increases in liver cholesterol concentrations in the mouse, rat, hamster and guinea pig ($P < 0.01$ in each instance).

Hyodeoxycholic acid, 1%. This bile acid caused a decrease in serum cholesterol concentration in the mouse ($P < 0.02$), but caused small increases in rat ($P < 0.01$) and hamster ($P < 0.02$). A considerable (40%) increase was observed in the guinea pig.

Hyodeoxycholic acid had little or no effect on liver cholesterol concentrations in any of the species.

Cholesterol 1%, cholic acid 0.5%, and hyodeoxycholic acid 1.0%. Hyodeoxycholic acid effectively prevented any liver or serum cholesterol accumulation in the mouse. It was very effective in preventing tissue cholesterol accumulation in the rat, although it did not maintain control concentrations in this species. In the hamster, hyodeoxycholic acid was partially effective in preventing cholesterol accumulation.

Lithocholic acid, 1%. This acid had no effect on mouse serum cholesterol concentrations, but caused a significant increase in the rat and doubled this concentration in the hamster.

In the liver, no effects were observed in the mouse and rat; a slight increase in liver cholesterol was noted in the hamster. At the 1% level, lithocholic acid is toxic to the hamster, causing gross hypertrophy of the bile duct system.

Chenodeoxycholic acid, 0.5%. This dietary supplement did not affect mouse serum cholesterol concentrations; it effected slight increases in the rat ($P < 0.01$) and guinea pig ($P < 0.01$), but doubled the concentration in the hamster. Chenodeoxycholic acid caused no significant changes in hepatic cholesterol concentrations, although a slight increase

($P < 0.01$) could be seen in the guinea pig. At the 0.5% level, this bile acid was toxic in the hamster and guinea pig, causing gross hypertrophy of bile duct and gall bladder.

Carcass cholesterol. Table 3 shows that, except in the hamster, none of the various dietary supplements caused changes in carcass cholesterol concentrations. Diets containing cholesterol or lithocholic acid caused small but significant increases in the hamster ($P < 0.01$ in each case). Hyodeoxycholic acid prevented this increase when added to the diet containing cholesterol and cholic acid.

Incorporation of acetate-1-C^{14}. Table 4 shows the effects of dietary supplements on the incorporation of acetate-1-C^{14} into serum and liver cholesterol one hour after intraperitoneal injection. The amounts of the isotope injected were proportional to the average weights of the various species.

Basal diet. Serum cholesterol-C^{14} concentrations in the rat and hamster were of similar magnitude. Almost no activity could be detected in guinea pig serum cholesterol. Mouse serum cholesterol-C^{14} concentrations were not determined due to an insufficient amount of sample.

The C^{14} activities of liver cholesterol in rat and hamster were approximately equal, and the highest in any of the different species. The activity of mouse liver cholesterol-C^{14} was about one-third that in rat and hamster. Very low counts were obtained in the β-sterol isolated from guinea pig liver.

Cholesterol-containing diets. These supplements inhibited incorporation of acetate-1-C^{14} into liver and serum cholesterol in all the species.

Corn oil. Corn oil had a small effect on serum cholesterol-C^{14} concentrations in rat ($P = 0.01$), but no significant effect in the hamster ($P = 0.16$). Hepatic β-sterol-C^{14} activity was unaffected in the mouse ($P = 0.6$) and rat ($P = 0.30$); however, in the hamster a somewhat lower C^{14} activity was found ($P < 0.01$).

Cholic acid. Cholic acid inhibited incorporation of acetate-1-C^{14} into liver cholesterol in all species studied. The largest effects were noted in mouse and hamster; a smaller effect in the rat. Changes in activities observed in serum of these species were similar to those in liver.

Hyodeoxycholic acid. Hyodeoxycholic acid caused significant increases in incorporation of acetate-1-C^{14} into cholesterol in mouse liver and the serum and liver of hamsters. There was only a slight increase in incorporation in the rat.

Lithocholic acid. Lithocholic acid failed to alter acetate-1-C^{14} incorporation in se-

TABLE 3

Effects of diets supplemented with bile acids or cholesterol, or both, on carcass cholesterol levels

Basal diet supplements	Mouse	Rat	Hamster	Guinea pig
		mg/gm carcass[1]		
None	2.94 ± 0.33[2]	1.87 ± 0.17	1.60 ± 0.19	1.93 ± 0.23
Corn oil, 3%	2.73 ± 0.25	1.77 ± 0.17	1.55 ± 0.21	—
Cholesterol, 1%	2.97 ± 0.31	1.79 ± 0.06	1.98 ± 0.11	1.82 ± 0.46
Cholesterol, 1% + corn oil, 3%	3.14 ± 0.65	1.89 ± 0.15	2.09 ± 0.24	—
Cholesterol, 1% + cholic acid, 0.5%	3.55 ± 0.24	1.89 ± 0.10	2.32 ± 0.36	1.43 ± 0.39
Cholesterol, 1% + cholic acid, 0.5% + hyodeoxycholic acid, 1%	2.71 ± 0.17	1.91 ± 0.17	1.87 ± 0.18	—
Cholic acid, 0.5%	3.34 ± 0.53	—	1.91 ± 0.24	1.30 ± 0.38
Hyodeoxycholic acid, 1%	2.99 ± 0.26	1.83 ± 0.12	1.81 ± 0.33	1.92 ± 0.28
Lithocholic acid, 1%	3.30 ± 0.55	1.97 ± 0.12	2.00 ± 0.24	—
Chenodeoxycholic acid, 0.5%	2.96 ± 0.43	1.83 ± 0.16	1.74 ± 0.32	2.13 ± 0.31

[1] Carcass weight indicates body weight minus weight of liver, blood and gastrointestinal tract content.
[2] SD.

TABLE 4

Effects of diets supplemented with bile acids or cholesterol, or both, on incorporation of acetate-1-C¹⁴ into serum, liver, and carcass cholesterol

Basal diet supplements	Serum cholesterol-C¹⁴			Liver cholesterol-C¹⁴				Carcass cholesterol-C¹⁴			
	Rat	Hamster	Guinea pig	Mouse	Rat	Hamster	Guinea pig	Mouse	Rat	Hamster	Guinea pig
	count/min/ml serum			*count/min/gm tissue*[1]				*count/min/gm tissue*[1]			
None	1128 ± 207[2]	1778 ± 656	6 ± 6	2145 ± 510	6940 ± 1055	7144 ± 1900	128 ± 73	472 ± 120	338 ± 32	427 ± 139	227 ± 72
Corn oil, 3%	861 ± 170	1318 ± 188	—	2000 ± 838	6349 ± 976	5429 ± 617	—	—	—	—	—
Cholesterol, 1%	69 ± 11	58 ± 47	0	45 ± 41	65 ± 58	0	0	—	—	—	—
Cholesterol, 1% + corn oil, 3%	60 ± 25	64 ± 34	—	0	0	0	—	—	—	—	—
Cholesterol, 1% + cholic acid, 0.5%	60 ± 28	134 ± 32	0	0	0	0	0	332 ± 49	108 ± 27	154 ± 30	70 ± 19
Cholesterol, 1% + cholic acid, 0.5% + hyodeoxycholic acid, 1%	38 ± 0	76 ± 31	—	185 ± 155	0	0	—	—	—	—	—
Cholic acid, 0.5%	333 ± 94	176 ± 75	0	65 ± 61	2590 ± 997	400 ± 353	0	—	—	—	—
Hyodeoxycholic acid, 1%	1299 ± 277	2840 ± 524	18 ± 5	3555 ± 466	9113 ± 1776	12084 ± 630	0	—	—	—	—
Lithocholic acid, 1%	1384 ± 341	2010 ± 713	—	3077 ± 1085	6928 ± 1400	6362 ± 2132	—	—	—	—	—
Chenodeoxycholic acid, 0.5%	595 ± 130	1541 ± 391	18 ± 1	315 ± 157	3276 ± 724	5330 ± 1452	0	—	—	—	—

[1] Wet weight.

[2] SD.

rum in the species investigated. This acid caused a significant increase in C^{14} incorporation in mouse liver ($P = 0.01$), but no change in the incorporation in the rat ($P = 0.90$) and hamster ($P = 0.50$).

Chenodeoxycholic acid. This bile acid inhibited acetate-1-C^{14} incorporation in mouse and rat liver and serum cholesterol. The inhibition was slightly smaller than that observed with the cholic acid supplement. Chenodeoxycholic acid did not inhibit cholesterol-C^{14} synthesis in the hamster.

Carcass acetate-1-C^{14}. Incorporation of acetate-1-C^{14} into carcass cholesterol appeared to be somewhat inhibited in all the species fed cholesterol plus cholic acid.

DISCUSSION

Experiments in comparative biochemistry clarify experimental differences observed among various species, and raise many provocative questions. A few of the more important observations of the present study are discussed below.

As expected, the responses of the various species to dietary cholesterol varied. In contrast to the mouse and rat, the hamster and guinea pig easily accumulated large amounts of serum and liver cholesterol when this sterol was added to the basal diet as the only supplement. In this respect the response of these species is similar to that of the rabbit (Cook, '59; Kritchevsky, '59). We have obtained serum cholesterol concentrations of 2,500 mg/100 ml in hamsters fed diets supplemented with cholesterol and cholic acid for three months.[3] However, in contradistinction to the rabbit, these animals did not develop aortic plaques. Bernick et al.[4] also reported difficulty in producing plaques in hamsters.

In general the mouse and rat have been assumed to respond similarly to dietary cholesterol. Moreover the investigations of Danielsson and Kazuno ('59) indicated that bile acid metabolism proceeds in an essentially similar manner in the two species. The present study shows that their responses to dietary cholesterol are in many respects similar. Although the rat accumulates cholesterol in both the liver and blood somewhat more readily than the mouse, a marked difference was noted in the response to the diet containing 1% cholesterol and 3% corn oil. Serum cholesterol concentrations in both rat and mouse remained at more or less the same concentrations as when the diet contained 1% cholesterol as the sole supplement, but liver cholesterol was considerably elevated in the rat, whereas mouse liver cholesterol remained almost constant. It appears that in the rat a considerable amount of liver cholesterol may accumulate before blood cholesterol is elevated. Ridout et al. ('54) have noted that increased liver cholesterol concentrations are sensitive indicators of cholesterol absorption in the rat.

When considering the comparative effects of dietary bile acids, one must recognize that the response of a species to any of these substances depends on three factors: (1) the bile acid administered; (2) the steroids resulting from bile acid metabolism in the liver of the species; and (3) the steroids resulting from bile acid metabolism by gastrointestinal tract bacteria. Therefore differences in response to bile acid supplements might be expected to result not only directly from species' differences but also from differences in the gastrointestinal bacterial spectrum when the basal diet is altered. For this reason it is desirable to maintain a constant basal diet in comparative studies of the effects of bile acids.

In general, differences in the response of the mouse and rat to bile acids is largely a matter of degree. However, one exception is that lithocholic acid causes increased blood cholesterol in the rat, but not in the mouse. Howe et al. ('60) have reported a depression of plasma cholesterol in mice treated with lithocholic acid.

Hyodeoxycholic acid is effective in lowering liver and blood cholesterol in the mouse (Beher et al., '59; Howe et al., '60). It was shown that the mechanism of this effect is a rapid excretion of fecal neutral sterols whose origin is the liver (Beher et al., '60). This results in decreased liver and blood cholesterol, which in turn

[3] Unreported data.

[4] Bernick, S., P. R. Patek, A. F. Wells and B. H. Ershoff 1962 Comparative effects of cholesterol feeding on thyroid gland, aortae and coronary arteries of rabbits, guinea pigs, hamsters and rats. Federation Proc., *21:* 101 (abstract).

causes an increased incorporation of acetate-1-C^{14} into liver β-sterols.

In the present experiments, although 1% hyodeoxycholic acid did not cause a reduction in blood and liver cholesterol in the rat and hamster, it caused an increase in the rate of incorporation of acetate-1-C^{14}. When fed together with cholic acid and cholesterol, this bile acid limited the accumulation of both serum and liver cholesterol in mouse, rat, and hamster. Howe et al. ('60) and Howe and Hutchison ('62) have also reported the limiting effect of hyodeoxycholic acid on mouse and rat serum cholesterol accumulation. The effects of hyodeoxycholic acid could not be studied satisfactorily in the guinea pig because of severe diarrhea.

As in previous experiments, hyodeoxycholic acid prevented any cholesterol accumulation in the mouse. Although mouse liver cholesterol was held to control concentrations by hyodeoxycholic acid, the incorporation of acetate-1-C^{14} was completely inhibited. It must be concluded that a measurable elevation in liver cholesterol level is *not* necessary for the feedback block of cholesterol biosynthesis. Although no definite conclusion can be reached, these data appear to support the hypothesis of Swell et al. ('58) that the concentration of recirculating cholesterol is the important factor in the control of liver cholesterol synthesis.

Under the dietary conditions of this experiment, lithocholic acid was ineffective and hyodeoxycholic acid was less effective in lowering tissue cholesterol concentrations in the mouse than in a previous study (Beher et al., '59). These differences in response are probably related to differences in composition of the basal diets used in the two studies. In the previous study, a highly purified, fat-free, high sucrose diet was used.

It has become increasingly clear that certain of the bile acids are important in controlling the rate of liver cholesterol synthesis through double feedback systems (Beher et al., '62; Myant and Eder, '61). If such systems are to be effective, they must utilize the bile acids normally present in the bile of the species. Thus one would expect both chenodeoxycholic acid and cholic acid to inhibit incorporation of acetate-1-C^{14} in rat liver cholesterol, and the data in table 4 show that this occurs. The same is true in the case of the mouse. However, chenodeoxycholic acid is ineffective in altering cholesterol biosynthesis in the hamster. If the hypothesis outlined above is correct, one would expect to find cholic acid but not chenodeoxycholic acid in hamster bile. We have investigated the bile acids in hamster bile by thin layer chromatography and found that it contains 95 to 98% cholic acid and a trace of dihydrocholanic acids, which are a mixture of deoxycholic acid and another unidentified acid.[5] Also chenodeoxycholic acid is toxic in the hamster.

Cholesterol metabolism in the guinea pig is strikingly different from that in the other species investigated. Guinea pig liver had an extremely slow rate of incorporation of acetate-1-C^{14}, which confirms the results of previous investigations (Nicholas and Thomas, '61; Schwenk et al., '55). Data in table 4 show that the rate of incorporation into extrahepatic tissues is about the same as that in the rat. Guinea pigs therefore might be expected to excrete a much larger proportion of metabolized cholesterol as neutral fecal sterols than as bile acids. This possibility is being investigated.

In general, carcass cholesterol-C^{14} concentrations were not determined, because a previous study (Beher et al., '59) showed that carcass C^{14} activity is affected by liver cholesterol C^{14} if the animals are killed later than 15 minutes after injection of the cholesterol precursor. Although the values presented indicate some inhibition of carcass cholesterol synthesis in the mouse, rat and hamster, fed cholesterol and cholic acid, this should not be considered as a true inhibition. Therefore, it is concluded that this diet does not inhibit carcass cholesterol-C^{14} synthesis in these species. In the case of the guinea pig, conditions are altered, since the rate of acetate-1-C^{14} incorporation into liver cholesterol is extremely slow in animals fed a basal diet, the activity of liver cholesterol-C^{14} would not contribute to the carcass C^{14} concentration. It can be concluded that the observed inhibition in the

[5] Unreported data.

guinea pig is a true inhibition of carcass cholesterol-C^{14} synthesis.

SUMMARY

Cholesterol concentrations in serum, liver and carcass, as well as acetate-1-C^{14} incorporation rates, were investigated in a comparative study on mice, rats, hamsters and guinea pigs, using a single standard basal diet supplemented in various ways with cholesterol and bile acids.

When diets were supplemented with cholesterol, guinea pigs and hamsters accumulated large quantities of this sterol, whereas mice and rats were more refractory.

In all species, cholesterol-containing diets inhibited incorporation of acetate-1-C^{14} into liver. None of the cholesterol or bile acid supplements had significant effects on carcass cholesterol concentrations in any of the species. Acetate-1-C^{14} incorporation into carcass remained unaffected in rat, mouse and hamster, but was inhibited in the guinea pig.

Those bile acids that are present in the bile of a given species inhibited incorporation of acetate-1-C^{14} into liver cholesterol. Other bile acids had varying effects on cholesterol concentrations and synthesis rates. Hyodeoxycholic acid limited the accumulation of liver and blood cholesterol of animals fed atherogenic diets.

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Effect of Lactose on Calcium Metabolism in Man[1]

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Many reports have appeared in the literature concerning the beneficial effects of lactose on the absorption of calcium from the gastrointestinal tract, most of the work having been done on experimental animals. The interpretation of the various studies is complicated by several factors, such as species differences, the age of the animals, and the amount of lactose used. Thus, French and Cowgill ('37) suggested that lactose was effective in increasing calcium absorption in young dogs and rats but not in fully grown animals. In some animal studies, the amount of lactose administered represented as much as 70% of the diet and had a marked laxative effect which interfered with the growth of the rats (French and Cowgill, '37; Outhouse et al., '37).

The conditions under which lactose is administered also affect the experimental results. Lengemann et al. ('57), as well as Wasserman and Comar ('59), demonstrated that lactose given simultaneously with tracer doses of Sr^{85} and Ca^{45} to fasting rats greatly increased the absorption of these radioisotopes. This effect was most marked when lactose and the radioisotopes were placed directly into the ileum. The effect was not obtained when lactose was injected into other parts of the intestine (Lengemann and Comar, '61). These investigators, as well as others, performed balance studies in rats to determine the effect of lactose (Bergeim, '26; Fournier et al., '55; Steggerda and Mitchell, '39). Outhouse et al. ('38) demonstrated in studies with vitamin D-deficient rats that lactose increased the ash content of bone. Roberts and Christman ('42) reported that lactose did not affect calcium absorption in rats. In their study rats were killed three hours after calcium and lactose were given by stomach tube, and the amount of calcium remaining in the gastrointestinal tract was used as a measure of calcium absorption.

The factors which play a role in the absorption of calcium from the intestine are poorly understood and the influence of lactose on calcium absorption under different conditions in man is not known. In view of the increased longevity of the population, the poor absorption of calcium by many older persons,[4] and the high incidence of osteoporosis, a search for a means of improving the absorption of calcium from the intestine appeared desirable. The results obtained with lactose in animals suggested that an increase in calcium absorption might be obtained by the use of this carbohydrate in man.

The present study on the effect of lactose on mineral and protein metabolism was therefore carried out on older persons in whom the absorption of calcium was low.

EXPERIMENTAL

The studies were performed under controlled dietary conditions in the Metabolic Research Ward on three ambulatory patients who were in good physical condition. The diagnoses, and the type and duration of the studies are listed in table 1.

Patient 1 was a 67-year-old white female with post-menopausal osteoporosis, body weight 68.2 kg, body height 157 cm. No physical abnormalities were present other than osteoporosis. Temperature, pulse, blood pressure, complete blood count, serum proteins and renal function were normal. The serum calcium ranged from 9.9 to 11.1 mg/100 ml, serum phosphorus from 2.9 to 3.5 mg/100 ml, and

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TABLE 1

List of patients studied

Patient	Age and sex	Diagnosis	Lactose	
			gm/day[1]	*days*
1	67 F	Mild osteoporosis	50	26
2	63 F	Osteoporosis	30	30
			20	24
3	50 M	Hypoparathyroidism	50	34
			20	18

[1] Given in three divided doses together with calcium supplements at meal time. Balances of calcium, phosphorus and nitrogen were performed on each patient in the control and lactose study. A tracer dose of Sr^{85} and Ca^{45} was given orally to patient 1 in the control and lactose study.

the serum alkaline phosphatase from 3.1 to 3.6 Bessey-Lowry units.

Patient 2 was a 63-year-old white female. Except for rounding of the dorsal spine, there were no physical abnormalities present. The roentgenograms of the skeleton showed marked osteoporosis. Body weight was 58.0 kg and body height 147 cm. Temperature, pulse, blood pressure, urinalysis, blood count, blood urea nitrogen, serum proteins and renal function were normal. Serum calcium ranged from 9.9 to 11.0 mg/100 ml, serum phosphorus from 2.5 to 4.1 mg/100 ml, and the alkaline phosphatase from 1.6 to 3.5 Bessey-Lowry units.

Patient 3 was a 50-year-old white male who had post-surgical hypoparathyroidism. A total thyroidectomy was performed for carcinoma of the thyroid 13 years prior to the study. At that time therapeutic doses of radioactive iodine were given which resulted in myxedema. The patient had been treated with thyroid extract, 0.0325 gm daily, for 13 years prior to the study and this medication was continued during the study. There was no evidence of recurrent carcinoma of the thyroid. Except for kyphoscoliosis and mild pulmonary emphysema, no physical abnormalities were noted. Body weight of the patient was 68.4 kg, body height 163 cm. Temperature, pulse, blood pressure, complete blood count, urinalysis, plasma proteins and renal function were normal. The serum calcium ranged from 7.5 to 8.7 mg/100 ml, serum phosphorus from 4.4 to 4.6 mg/100 ml, and the serum alkaline phosphatase from 3.9 to 5.8 Bessey-Lowry units.

The diet was kept constant throughout the study and contained 1850 Cal., 265 gm carbohydrate, 59 gm fat, 68 gm protein, 152 mg calcium and 707 mg phosphorus /day. The composition of the diet is listed in table 2. The calcium intake was increased to approximately 1500 mg/day by adding calcium gluconate tablets to the low calcium diet; all other constituents of the diet remained unchanged. The daily fluid intake was kept constant and the body weight of the patient was determined daily. All urine and stool specimens were collected from the start of the study. The fluid intake, the urine volume, the urinary excretion of creatinine, calcium and phosphorus were determined daily. The studies were divided into control and experimental periods. Metabolic balances of calcium, phosphorus and nitrogen were determined on each patient on aliquots of 6-day pools of urine and stool and on aliquots of the diet. The duration of the control studies ranged from 16 to 42 days. In the experimental studies, the patients received from 20 to 50 gm lactose/day. One-third of the daily dose of lactose and of the calcium supplement was given simultaneously by mouth with each meal. The duration of the experimental studies ranged from 18 to 34 days. The metabolic effect of lactose was studied twice in patient 2. During the second study, in both the control and lactose periods, this patient received 50 mg of a synthetic weakly estrogenic hormone, 3-methoxy-16α-methyl-1,3,5 (10) estratriene-16β, 17β-diol,[5] intramuscularly/day.

The diet and excreta were analyzed for nitrogen, calcium and phosphorus. Ni-

[5] Mytatrienediol, G. D. Searle & Company, Chicago.

TABLE 2

Composition of metabolic low calcium diet

Carbohydrate		Protein		Fat	
	gm/day		*gm/day*		*gm/day*
200 ml Fruit juice	24	100 gm Bread		50 gm Butterfat	41
20 gm Rice[1]	16	20 gm Spaghetti[1]	15	20 gm Cream	2
100 gm Bread	52	20 gm Rice[1]		175 gm Meat	24
70 gm Jelly	46	175 gm Meat			
20 gm Spaghetti[1]	15	(beef and turkey)	40		
100 gm Potatoes[2]	19	100 gm Potatoes[2]			
200 gm Canned		200 gm Canned	5		
vegetables	18	vegetables			
225 gm Sweetened					
canned fruit	44				
28 gm Sugar	28				
Total	264		60		67

[1] Weight of raw food.
[2] Weight of cooked food.

trogen was determined by the Kjeldahl method, calcium by the method of Shohl and Pedley ('22) and phosphorus by the method of Fiske and SubbaRow ('25). Stool calcium and phosphorus were determined on dry-ashed aliquots of 6-day metabolic pools.

A single tracer dose of both Sr^{85} and Ca^{45} was given simultaneously by the oral route to patient 1 in the control and lactose study. Plasma levels and urinary and fecal excretions of both radioisotopes were determined in each study. On the first day of the study, plasma was obtained at 1, 4, 8 and 24 hours for Sr^{85} and Ca^{45} analyses. Subsequently, the plasma levels of both radioisotopes were determined daily for 6 days and three times per week thereafter. On the first day of the radioisotope studies the urine output was divided into fractional collections at the time blood samples were obtained, for Sr^{85} and Ca^{45} analyses. For the remainder of the study, radioassays and calcium determinations were performed daily on 24-hour urine collections. Plasma samples were obtained daily for 6 days and three times per week thereafter. Each stool specimen was analyzed separately for calcium, Sr^{85} and Ca^{45}. The duration of the tracer studies was 17 and 22 days in the control and lactose study, respectively.

The oral administration of the tracers Ca^{45} and Sr^{85} and the counting techniques for both radioisotopes have been previously described (Spencer et al., '60). The Sr^{85} was counted in a well-type scintillation counter; Ca^{45} was counted after precipitation with carrier calcium as the oxalate in a Q-gas flow counter.

RESULTS

The calcium, phosphorus and nitrogen balances of the three patients studied are shown in table 3. Lactose did not improve the calcium balance of patient 1 who received 50 gm lactose/day for 26 days. Similarly, the calcium retention of patient 2 did not increase when 30 gm lactose were given daily for 30 days (first study), but a very slight increase in calcium retention was noted in this patient when 20 gm lactose were given daily for 24 days (second study). In patient 3, the average balance data indicated that the calcium balance improved by 212 mg/day when 50 gm lactose were given daily for 34 days (first study), whereas the calcium balance during the second lactose study was similar to that of the control. However, figure 1 shows that the fecal calcium excretion of patient 3 fluctuated greatly from period to period and that the improvement of the calcium balance in the first lactose study of this patient was only slight. Great fluctuations in fecal calcium excretion were also noted in the second lactose study.

The urinary calcium excretion of the three patients did not change according to any consistent pattern during the administration of lactose (table 3). There may have been a slight decrease in the urinary calcium excretion of patient 1. No con-

TABLE 3

Calcium, phosphorus and nitrogen metabolism of three patients receiving lactose

Study		Body wt	Calcium				Phosphorus				Nitrogen			
			Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance
	days	*kg*	*mg/day*			*mg/day*	*mg/day*			*mg/day*	*mg/day*			*mg/day*
						Patient 1								
Control	42	68.3	1496	224	1152	+120	681	442	224	+15	11172	8895	1200	+1077
Lactose, 50 gm/day	26	68.7	1488	206	1253	+ 29	649	313	257	+79	10489	7494	1409	+1586
						Patient 2								
Control	18	58.0	980	92	867	+ 21	818	511	299	+ 8	12729	10400	1650	+ 679
Lactose, 30 gm/day	30	59.1	973	85	889	− 1	836	467	316	+53	13189	10061	1895	+1233
Control[1]	16	56.1	856	41	746	+ 69	645	252	317	+76	11341	8953	1434	+ 954
Lactose[1], 20 gm/day	24	56.3	865	47	715	+103	672	316	313	+53	11237	9213	1491	+ 533
						Patient 3								
Control	24	58.4	1553	58	1517	− 22	634	302	343	−11	11052	9124	1274	+ 654
Lactose, 50 gm/day	34	57.7	1565	60	1315	+190	681	288	320	+73	11186	8843	1558	+ 785
Control[2]	18	57.1	1579	86	1335	+158	704	324	329	+51	11451	9161	1459	+ 831
Lactose[2], 20 gm/day	18	55.9	1577	85	1369	+123	702	344	335	+23	11447	8657	1333	+ 457

[1] Second control and lactose study was performed 5 months after the first lactose study. During this time interval, the patient received 3-methoxy-16α-methyl-1,3,5(10 estratriene-16β,17β-diol) (Mytatrienediol), which was continued during the control and lactose study. Mytatrienediol is a synthetic weak estrogenic compound.

[2] Time interval between first and second lactose study was 8.5 months.

sistent change of the phosphorus balances was noted except in the first study of patient 3 in whom the changes in phosphorus balance corresponded to those of the calcium balance (first study). No consistent changes of the nitrogen balances were noted in the three patients studied.

Table 4 shows that the daily urinary Sr^{85} and Ca^{45} excretion of patient 1 was

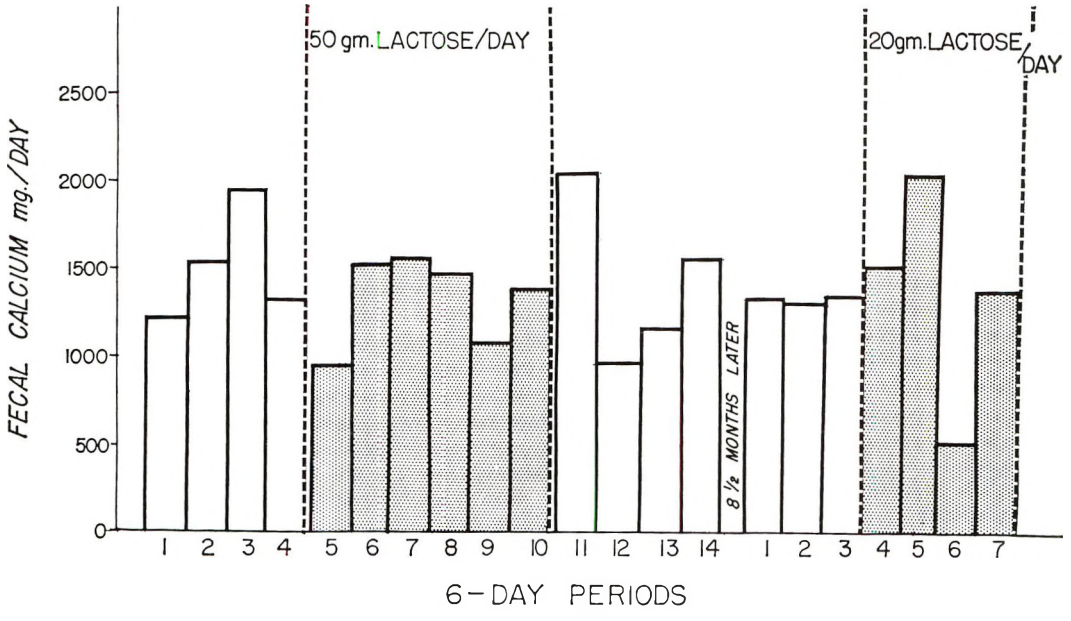

Fig. 1 Effect of lactose on fecal calcium excretion (patient 3).

TABLE 4

Effect of lactose on urinary Sr^{85} and Ca^{45} excretion in man (patient 1)[1]

Study day	% Administered dose				Urinary calcium	
	Sr^{85}		Ca^{45}		Control	Lactose[2]
	Control	Lactose[2]	Control	Lactose[2]		
					mg/day	*mg/day*
1	4.0	3.9	2.2	1.8	258	196
2	2.0	1.8	1.2	1.1	191	170
3	1.2	1.3	1.0	0.9	206	169
4	1.0	1.2	0.8	1.0	192	195
5	0.7	0.9	0.7	0.7	229	181
6	0.6	0.7	0.6	0.7	250	206
7	0.4	0.6	0.5	0.6	222	222
8	0.5	0.5	0.5	0.5	252	198
9	0.3	0.5	0.4	0.5	224	255
10	0.2	0.4	0.4	0.3	205	172
11	0.2	0.2	0.4	0.3	260	202
12	0.1	0.2	0.2	0.3	183	184
13	0.1	0.2	0.2	0.2	170	172
14	0.1	0.2	0.2	0.2	240	267
15	0.1	0.1	0.2	0.2	216	181
				Average	221	198

[1] A tracer dose of Sr^{85} and Ca^{45} was given orally on the first day of the control and experimental study.

[2] 50 gm lactose were given daily in three doses.

similar in the control and lactose studies. The urinary calcium excretion was somewhat lower on some days of the lactose intake. The average urinary calcium excretion was 220 mg and 198 mg in the control and lactose phase, respectively.

Table 5 shows data on urinary and fecal Sr^{85} and Ca^{45} excretions. The cumulative urinary excretion of Ca^{45} was lower than of Sr^{85} in both the control and lactose studies and no changes in the excretion of either radioisotope were noted during administration of lactose. The fecal excretions of both Sr^{85} and Ca^{45} were somewhat lower in the lactose study than in the control phase indicating some improvement in calcium and strontium absorption.

Figure 2 shows the plasma levels of Ca^{45} and Sr^{85} of patient 1 in the control and lactose studies. The plasma level of

TABLE 5

Urinary and fecal Sr^{85} and Ca^{45} excretion before and during lactose administration (15-day cumulative excretions)[1]

Study	Urinary excretion			Stool	
	Sr^{85}	Ca^{45}	Calcium	Sr^{85}	Ca^{45}
	% dose	*% dose*	*mg/day*	*% dose*	*% dose*
Control	11.9	9.6	220[2]	84.0	81.4
Lactose[3]	12.1	9.3	198[2]	75.6	72.0

[1] Sr^{85} and Ca^{45} were given simultaneously as a single tracer dose by the oral route on the first day of each study. The excretions are cumulative for 15 days.
[2] Average of 15 days.
[3] 50 gm lactose given per day in divided doses.

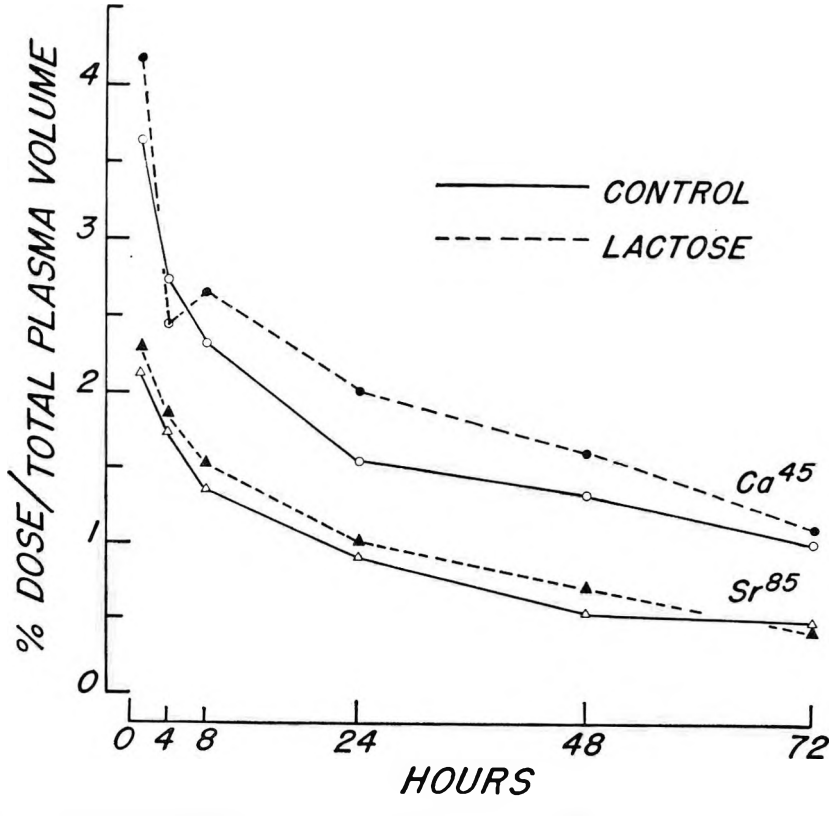

Fig. 2 Sr^{85} and Ca^{45} plasma levels before and during administration of lactose in man.

Ca^{45} was higher than that of Sr^{85} in both study phases. During the administration of lactose, the plasma level of Ca^{45} was higher for the first 48 hours following the administration of the radioisotopes than in the control study. Similarly, there was a slight increase of the plasma level of Sr^{85} in the lactose phase for the same period of time. The higher Ca^{45} and Sr^{85} plasma levels are in agreement with the lower fecal Ca^{45} and Sr^{85} excretion.

DISCUSSION

In the studies presented, the effect of lactose on the improvement of calcium absorption could not be detected with any certainty by the data obtained with metabolic balances. The fecal calcium excretions were similar to that of the control in patients 1 and 2 in the lactose study. The stool calcium of patient 3 was decreased by 200 mg/day in the first lactose study, indicating some improvement of calcium absorption. However, in the subsequent control study the stool calcium was also lower by 200 mg/day, which may have been due to a change in metabolism of this patient. The difficulty in interpreting such data is illustrated graphically in figure 1. Inaccuracies in the separation of stool collections of the various metabolic periods occur due to delay or irregularity in the passage through the gastrointestinal tract, and the appearance of the stool marker may not correspond exactly to the food intake of the corresponding study period. Lowering of the fecal calcium excretion in any single metabolic period is therefore of no significance. It was hoped that the effect of lactose on calcium absorption would be sufficiently great to be detected unequivocally by the calcium balance data. However, these data indicate that some effect of lactose cannot be ruled out especially in the first study of patient 3. A more definite effect of lactose might have been more easily detected by this technique in patients of different age groups or possibly in patients in different metabolic states.

Although the calcium metabolism of elderly persons may be expected to differ from that of young children, it remains of interest to compare the balance data with those of Mills et al. ('40). These investigators performed a study on children of the comparative effect of lactose with that of other sugars. In these studies a daily dose of 36 gm lactose was given to boys from 5 to 7 years of age and a slight improvement in calcium retention was noted. However, statistically this effect was not quite significant at the 5% level, and the improvement in balance was almost entirely due to a decrease in urinary calcium excretion. In the present lactose study, the urinary calcium excretion did not decrease.

In contrast to the metabolic data, the radioisotope data of patient 1 indicate a slight increase in the absorption of both Ca^{45} and Sr^{85}, although the magnitude of the increase is uncertain. The plasma levels of both radioisotopes in the lactose phase suggest an increase in absorption of possibly 10%, whereas the data of the fecal Ca^{45} and Sr^{85} excretion indicate a much greater increase. If the net absorption of the radionuclides is calculated as 100% minus the percentage of the dose excreted in feces, the absorption of Ca^{45} is 18.6 and 28.0% in the control and lactose study, respectively, and 16.0 and 24.4% for the absorption of Sr^{85} for the corresponding two study phases. The values both for Ca^{45} and Sr^{85} represent an increase in absorption greater than 50%. However, stool analyses are subject to a standard deviation of approximately 5%, as determined from numerous studies in our own laboratory, so that the 95% confidence limits for the increase in absorption range from less than 0% to more than 100%.

In cases of low absorption of radioactive calcium or strontium, the plasma levels of the radioisotopes appear to be more accurate indicators of the true absorption than stool analyses.[6] The change in plasma levels is also a more reliable reflection of changes in absorption under different experimental conditions. The best estimate of the increase of calcium absorption is therefore about 10%, and this figure applies only to the absorption of Ca^{45} and Sr^{85} with a *single meal* (breakfast) with which the radioisotopes were ingested. The lack of decrease in stool calcium excretion in the lactose phase may be due to increased

[6] Samachson, J., and H. Spencer 1960 Significance of comparative Sr-85 and Ca-45 plasma values in man. Federation Proc., *19*: 250 (abstract).

excretion of endogenous fecal calcium while improving calcium absorption, although this is less likely.

SUMMARY

1. The effect of lactose on the absorption and excretion of calcium was studied in three patients under controlled dietary conditions in the Metabolic Research Ward. Simultaneous Ca^{45} and Sr^{85} absorption studies were carried out in one of the patients.

2. Lactose given in amounts up to 50 gm/day did not decrease the urinary excretion of calcium nor did it improve the calcium balance. No consistent effect on phosphorus or nitrogen metabolism was noted.

3. The radioisotope studies carried out in a single patient indicate that the absorption of Ca^{45} and Sr^{85} was improved slightly in the lactose phase.

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