UTILIZATION OF A "SYNTHETIC" TRIGLYCERIDE PREPARATION BY WEANLING RATS[1, 2]

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ONE FIGURE

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The fact that retardation in growth occurred when rats and other animals were fed diets deficient in essential fatty acids or free from neutral fats has been reviewed by Burr ('42), Burr and Barnes ('43) and by Deuel ('51). Deuel et al. ('50) suggested that the beneficial effect of fat in the diet cannot be explained entirely by its essential fatty acid content, but must be traced to some additional factor or factors. They showed that when 20 mg of methyl linoleate was given daily as a supplement to rats on a fat-free diet, a prompt response in growth resulted. However, the animals soon ceased to grow, and growth was not augmented by an increase of methyl linoleate to 60 mg daily, but the feeding of a diet containing 10% cottonseed oil resulted in a further acceleration of gain in weight. In another communication, the same group of workers (Greenberg et al., '50) reported that greater growth was found in female rats fed 30% cottonseed oil diets than in those on a fat-low regimen supplemented with an optimum

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[2] Part of the preliminary results has been presented at the symposium on Fat Metabolism sponsored by the M. & R. Laboratories, Feb., 1954.

quantity of methyl linoleate. In a later study they (Greenberg et al., '51) further demonstrated that the gain in weight was greater in both male and female rats receiving a diet containing 30% cottonseed oil than in those on a fat-low regimen supplemented with as much as 100 mg of linoleic acid, 20 mg of methyl arachidonate or a combination of these two essential fatty acids. Meng and Youmans ('53) showed that in dogs receiving isocaloric amounts of food by parenteral alimentation, only those getting emulsified neutral fat (triglyceride) gained weight. The addition of adequate amounts of methyl esters of linoleic, linolenic and arachidonic acids to the food of the dogs receiving no neutral fat prevented the pathological changes of the hair and skin but did not correct the weight loss.

In the present work a synthetic triglyceride was used. The experiments were designed to determine whether or not the synthetic preparation with known fatty acid composition but free from any impurities or other unknown factors can be utilized by weanling rats as well as natural edible fat (lard) and to investigate further the mechanism relative to the superior nature of neutral fats over essential fatty acids in promoting growth. The changes in weight, nitrogen balance, water balance, body composition and fecal fat excretion have been used as criteria.

EXPERIMENTAL

Weanling male rats of the Sprague-Dawley strain and weighing from 40 to 45 gm were divided into groups of 6 each. The individual groups were fed the following diets: I. fat-free, II. 5% lard, III. 25% lard, IV. 5% synthetic triglyceride, and V. 25% synthetic triglyceride. The composition of the diets is shown in table 1. The synthetic triglyceride [3] was prepared by esterification of pure fatty acids with glycerol. Its fatty acid composition which resembles that of human body depot fat consisted of oleic acid, 60%; palmitic acid, 30%; stearic

[3] Kindly supplied by the Oilseed Division, Southern Regional Research Laboratory, Department of Agriculture, New Orleans.

acid, 7.4%; and linoleic acid, 2.6%, with an iodine number ranging from 51 to 55 and hydroxyl value of 2.19. The free fatty acid of the preparation varied from 0.02 to 0.15% as oleic acid and the peroxide value was 0.

Two rats of the same dietary group were placed in one metabolic cage. Water and food were allowed ad libitum with amounts consumed carefully measured. Both urine and feces

TABLE 1

Composition of diets

DIETARY COMPONENT	FAT CONTENT				
	Fat-free	Lard 5%	Lard 25%	Synthetic triglyceride 5%	Synthetic triglyceride 25%
Casein,[1] *gm*	30	30	30	30	30
Sucrose, *gm*	61	56	36	56	30
Lard, *gm*	0	5	25	0	0
Synthetic triglyceride, *gm*	0	0	0	5	25
Salt mixture,[2] *gm*	4	4	4	4	4
Roughage,[3] *gm*	3	3	3	3	3
Yeast, dry, *gm*	2	2	2	2	2
Vitamin supplements					
Water-soluble vitamin mixture,[4] *gm*	0.3	0.3	0.3	0.3	0.3
α-Tocopherol,[5] *mg*	5	5	5	5	5
Vitamin A (U.S.P. units)	2,750	2,750	2,750	2,750	2,750
Vitamin D[6] (U.S.P. units)	396	396	396	396	396
Calculations					
Per cent calories from fat[7]	0	11.6	46.2	11.6	46.2
Calories/gm	3.73	3.99	5.03	3.99	5.03

[1] Vitamin-free, LABCO.

[2] Wesson salt mixture (Wesson, '32).

[3] Ruffex.

[4] Water-soluble vitamin mixture as used by Deuel et al. ('47).

[5] α-Tocopherol acetate, Merck & Co.

[6] Vitamins A and D were furnished as Oleum Percomorphum, Mead Johnson and Co.

[7] Assuming that lard and synthetic triglyceride each furnished 9.3 Cal./gm.

of the rats in the individual dietary groups were collected daily and combined into weekly pools. The rats were weighed weekly. The experiment was carried out for 13 weeks after which two rats from each group were taken at random and were killed by decapitation. After blood was drained, the rat was weighed and the carcass was ground several times through a meat grinder. Twenty or 50 gm of wet tissue were dried in a vacuum at low temperature. The dry tissue was again weighed and body water content was obtained by difference. The dried tissue was extracted with diethyl ether for 36 hours in a Soxhlet apparatus. The ether extract was then transferred to a volumetric flask. An aliquot of this extract was used for the determination of body total lipids. The fat-free tissue residue was ground and used for nitrogen determination by the Kjeldahl method. The fecal and urinary nitrogen were determined by the semi-micro Kjeldahl techniques. Fecal fat was determined by extracting an aliquot of ground feces three times with 95% ethyl alcohol and three times with diethyl ether. The alcohol-ether extract was dried and re-extracted with petroleum ether. The petroleum ether extract was dried and weighed. Wijs' method was used for determining the iodine number of the body lipids. The water balance was measured by the differences between the volume of water intake by mouth and the volume of urinary output.

RESULTS

Changes in body weight and general appearance. The average body weight changes for all 5 groups of rats are shown in figure 1. Two things are demonstrated: (1) rats fed the diet with no added fat failed to gain as much weight as those on diets containing lard or synthetic triglyceride; (2) there was no difference in the growth curves between the rats of the 25% lard and the 25% synthetic triglyceride groups.

It can be seen in table 2 that the average daily gain in body weight per rat in the fat-free group was 2.49 gm, a value which is 14 to 28% lower than the weight gain of the groups

receiving fat in the diet. The differences in average weight gain between the fat-free group and the 5% lard, 25% lard, 5% synthetic triglyceride and 25% synthetic triglyceride groups were 0.69, 0.96, 0.42 and 0.78 gm respectively, with p values of < 0.01, < 0.01, < 0.02 and < 0.001 respectively

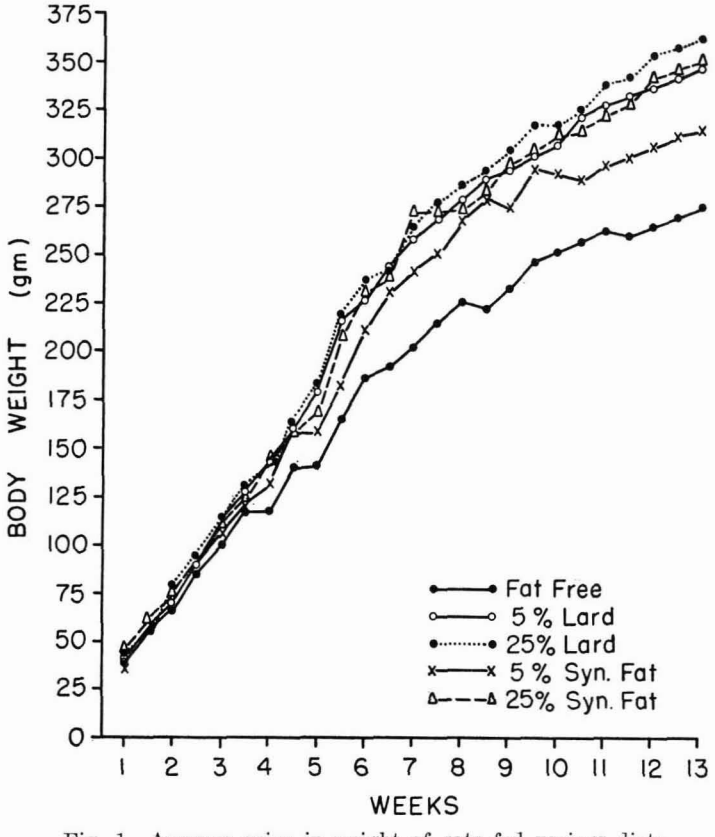

Fig. 1 Average gains in weight of rats fed various diets.

which are statistically significant. However, rats fed the fat-free diet consumed a greater amount of food (9.92 gm/100 gm rat/day) resulting in a slightly higher caloric intake (37.05 cal./100 gm rat/day). The average caloric intake (per 100 gm rat per day) required per gram of daily weight gain was 14.9 for the fat-free group. The values were 11.3, 10.6, 11.7 and

TABLE 2

Average food and caloric intake and daily gain in body weight

DIET	NO. OF RATS	FOOD INTAKE/100 GM RAT/DAY				CALORIC INTAKE/100 GM RAT/DAY				AVERAGE DAILY GAIN IN BODY WT./RAT	CALORIES REQUIRED/GM GAIN/100 GM RAT/DAY
		Total	Fat	Protein	Carbohydrate	Total	From fat	From protein	From carbohydrate		
		gm	*gm*	*gm*	*gm*					*gm*	
Fat-free	6	9.92	....	2.976	6.055	37.05	....	12.22	24.84	2.49 ± 0.05	14.9
5% lard	6	9.08	0.456	2.723	5.055	36.13	4.20	11.20	20.73	3.18 ± 0.09	11.3
25% lard	6	7.28	1.820	2.188	2.635	36.69	16.94	8.97	10.79	3.45 ± 0.16	10.6
5% synthetic triglyceride	6 [1]	8.39	0.423	2.565	4.697	33.95	3.87	10.55	19.27	2.91 ± 0.11	11.7
25% synthetic triglyceride	6	7.28	1.818	2.182	2.631	36.58	16.93	8.90	10.73	3.27 ± 0.09	11.2

[1] Six rats were used during the one- to 7-week period, 5 during the 8- to 13- week period.

TABLE 3

Average nitrogen and water balance

DIET	NO. OF RATS	NITROGEN/100 GM RAT/DAY							WATER/100 GM RAT/DAY [1]			
		Intake	Urinary nitrogen		Fecal nitrogen		Balance		Intake	Urinary output	% of intake excreted as urine	Balance
			Am't.	% of intake	Am't.	% of intake	Am't.	% of intake				
		mg	*mg*		*mg*		*mg*		*ml*	*ml*		*ml*
Fat-free	6	477	152	31.9	16	3.4	+ 309	64.2	18.99	3.59	18.9	+ 15.40
5% lard	6	436	126	28.9	11	2.5	+ 297	68.1	15.29	3.55	23.2	+ 11.74
25% lard	6	351	92	26.2	8	2.3	+ 251	71.5	13.98	2.88	20.6	+ 11.10
5% synthetic triglyceride	6 [2]	412	123	29.9	11	2.7	+ 278	67.5	15.05	3.40	22.6	+ 11.65
25% synthetic triglyceride	6	349	100	28.7	6	1.7	+ 243	70.0	13.63	2.85	20.9	+ 11.78

[1] Measured by the difference between water intake by mouth and urinary output. Water loss by route other than kidney was not considered.

[2] Six rats were used during the one- to 7-week period, 5 during the 8- to 13-week period.

11.2 for 5% lard, 25% lard, 5% synthetic triglyceride and 25% triglyceride groups respectively.

Rats fed the fat-free diet not only demonstrated retarded growth but also developed pathologic changes in the hair and skin characteristic of essential fatty acid deficiency. No such findings were observed in rats fed lard or synthetic triglyceride diets.

Nitrogen balance. The nitrogen intake of the rats fed fat-free diets was higher than that of all other groups with 477 mg/100 gm rat/day, and the average nitrogen balance was + 309 mg/100 gm rat/day. The balances for the other groups were: 5% lard, + 297; 25% lard, + 251; 5% synthetic triglyceride, + 278; and 25% synthetic triglyceride, + 243 mg. Both urinary and fecal nitrogen excretion were higher in the fat-free group. As the result of higher nitrogen excretion, only 64.2% of the total nitrogen intake was retained. The nitrogen retentions in the 5% lard, 25% lard, 5% synthetic triglyceride and 25% triglyceride groups were 68.1, 71.5, 67.5, and 70.0% (per 100 gm rat/day) respectively. The results are shown in table 3.

Water balance. Table 3 also shows the water balance of rats fed various diets. The water intake was considerably higher for the group receiving the fat-free diet than that for the other groups. The water intake of rats fed diets containing 5% lard or 5% synthetic triglyceride was slightly higher than that for those on 25% fat diets. The volume of urinary output of the fat-free, 5% lard, and 5% synthetic triglyceride groups was approximately the same. However, the water excreted as urine expressed as the percentage of intake was considerably lower in the fat-free group (18.9%, fat-free group; 23.2%, 5% lard group; and 22.6%, 5% synthetic triglyceride group). The 25% fat groups excreted less water as urine both in terms of absolute quantity and percentage of the intake (20.6%, 25% lard, and 20.9%, 25% synthetic triglyceride). The water balance, measured by the difference between the intake and the urinary output, for the rats fed the fat-free diet, was considerably higher (+ 15.4 ml/100 gm

rat/day). The values for 5% lard, 5% synthetic triglyceride, 25% lard and 25% synthetic triglyceride were + 11.74, + 11.65, + 11.10 and + 11.78 ml/100 gm rat/day respectively.

Body composition. The results of carcass analysis of two rats selected at random from each of the dietary groups are shown in table 4. It can be noted that the water content was remarkably uniform, ranging from 59.5 to 63.0% regardless of the fat content in the diets. Likewise, the body protein

TABLE 4

Body composition based on two animals from each group

DIET	RAT NO.	LIPID		PROTEIN	WATER
		%	*iodine no.*	%	%
Fat-free	2	15.1	65.2	19.1	60.0
	6	14.8	67.6	18.9	61.0
5% lard	7	16.3	59.8	18.2	60.6
	9	16.2	65.4	18.5	59.6
25% lard	13	20.7	75.8	19.1	59.0
	14	16.9	79.8	19.0	60.0
5% synthetic triglyceride	19	15.2	70.5	17.1	59.5
	24	17.5	67.7	21.6	60.0
25% synthetic triglyceride	25	16.5	72.5	21.0	63.0
	30	20.8	71.6	17.0	59.5

TABLE 5

Fecal fat

DIET	NO. OF RATS	EXPER. PERIOD	TOTAL FECES EXCRETED	FAT IN FECES	TOTAL FAT EXCRETED	FAT/100 GM RAT/DAY	INGESTED FAT RETAINED
		weeks	*gm*	%	*gm*	*gm*	%
Fat-free	6	13	364.7	5.1	18.7	0.0200	....
5% lard	6	13	312.2	7.9	21.6	0.0190	96.1
25% lard	6	13	410.4	9.6	39.3	0.0340	98.1
5% synthetic triglyceride	6 [1]	13	329.1	7.2	23.7	0.0230	94.6
25% synthetic triglyceride	6	13	276.9	13.7	37.9	0.0250	98.6

[1] Six rats were used during the one- to 7-week period, 5 during the 8- to 13-week period.

was of the same pattern (from 17.1 to 21.6%). The values for total lipids of the fat-free group were slightly lower than those of the rats fed diets containing 25% fats. The iodine number of the body lipids of the rats in the high fat groups was higher than that of the fat-free group.

Excretion of fat in feces. On the basis of fat/100 gm rat/day, animals fed the fat-free diet excreted as much fat in the feces as those of the 5% fat groups. Rats fed 25% fat diets showed higher excretion, especially the lard group. The values were 0.020, 0.019, 0.034, 0.023, and 0.025 gm/100 gm rat/day for fat-free, 5% lard, 25% lard, 5% synthetic triglyceride and 25% synthetic triglyceride groups respectively. The amounts of ingested fat which were retained varied from 94.6 to 98.6%. Diarrhea was never observed in rats fed diets containing lard or synthetic triglyceride. The results are shown in table 5.

DISCUSSION

Our results confirm the findings observed by other investigators that fat is essential for optimum growth in rats. It may be further noted that the synthetic triglyceride preparation was biologically utilized and was apparently equivalent to lard as a dietary constituent. This is true at least when it is incorporated in the diet at the higher level. Failure of rats fed 5% synthetic triglyceride diet to gain as much weight as those on a 5% lard regimen might have been due to their lower caloric intake (table 2) or to insufficient intake of linoleic acid, or both. Rats of the 5% synthetic triglyceride group consumed 0.423 gm of fat/100 gm rat/day (table 2), which supplied approximately 11 mg of linoleic acid. According to Deuel et al. ('50), Greenberg and co-workers ('50, '51), and others, the optimum intake of linoleate ranged from 40 to 100 mg per day. Lard, on the other hand, contains a greater amount of linoleic acid (Hilditch, '40) which may have been sufficient. However, it should be pointed out that the difference in average daily weight gain between rats fed 5% synthetic triglyceride and those on 5% lard diet is only

0.27 gm which is not statistically significant. Nevertheless, the differences in weight gain between rats of the 5% synthetic triglyceride group and those of the 25% fat groups are statistically significant while those between the 5% lard group and the 25% fat groups are not. It is possible that a difference in growth between rats in the 5 and 25% lard groups might have been observed if a large number of animals had been used or the observations made over longer periods of time or both. However, it may be pointed out that Hoagland et al. ('52) also failed to show any statistically significant differences in weight gain between rats fed diets containing 5.0, 10.98, and 18.27% lard.

Our results indicate that more calories are required per gram of daily weight gain in rats on a fat-deficient diet than in those with liberal fat intake (table 2). It seems reasonable to state that neutral fat in the diet apparently favors a more efficient utilization of calories since the rats on the fat-free diet showed the poorest growth despite the highest caloric intake. One of the explanations offered by Greenberg et al. ('51) for the more satisfactory effect of the fat as compared with the fat-low linoleate-supplemented regimen, was that diet containing fat is more efficient in utilization of the food. Sinclair ('52) reported similar results in both pair-fed rats and ad libitum-fed litter mates and suggested that either inefficient metabolism is occurring in the fat-deficient rat or its metabolic rate is greatly increased. Fenton and Carr ('51) also demonstrated that the efficiency of food utilization for body weight gain increased with increasing dietary fat levels. It is likely that incorporation of neutral fat in the diet may conserve energy which is required for its conversion from carbohydrate or protein. The complex enzymatic reactions involved in the processes of fatty acid synthesis have been reviewed by Chaikoff and Brown ('54) and the details will not be further emphasized. However, it may be pointed out that another biological analogy can be cited:

The relative effectiveness of the essential fatty acids with regard to their biological activity when fed to fat-deficient

rats is linolenate < linoleate < arachidonate (Greenberg et al., '51). Turpeinen ('38) and Smedley-MacLean and Nunn ('40) concluded that the principal unsaturated fatty acid required by the body is arachidonic acid. It has been demonstrated by Nunn and Smedley-MacLean ('38), Barki and co-workers ('49), Rieckehoff and associates ('49), Widmer and Holman ('50) and Holman ('51) that the interconversion of linoleic and linolenic acids to arachidonic acid has occurred in rats. The reason arachidonic acid has a greater biological activity than linoleic and linolenic acids is probably that the former can be utilized as such whereas the latter two have to be converted to arachidonic acid which requires energy and involves specific enzymatic processes. Thus, it may be postulated that two factors should be considered concerning the importance of fat in nutrition: (1) its content of essential fatty acids and the amount consumed, and (2) the quantity of neutral fat ingested.

The water consumption by our animals was similar to that reported by Burr and Burr ('30) and by Ramalingaswami and Sinclair ('51) in that the rats fed the fat-deficient diet consumed an increased amount. It is further noted that the volume of water consumption of rats on various diets varies inversely with the levels of dietary fat (table 3). Water formed as the result of oxidation of foodstuffs did not vary significantly between rats on various diets (table 6). Since the urinary output of fat-deficient rats is approximately the same as that of the rats on 5% fat, and only slightly higher than that of the 25% fat groups, the high water "retention" of the deficient rats seems apparent. However, measurement of the body water content of rats on various diets shows no significant difference (table 4). This indicates that increase in water loss through routes other than the kidney must have occurred in the fat-deficient rats. Ramalingaswami and Sinclair ('51) showed that increase of water loss through the skin occurs in the fat-deficient rats. They concluded that the loss of water through the skin of deficient rats is attributable to the increase of permeability.

Only two rats from each of the various dietary groups were used for analyses of the body composition. However, the values for body water, protein and lipid yielded by them are in accordance with those reported by Deuel et al. ('44) and Scheer and associates ('47). The increased body lipid content of rats on diets providing liberal fat intake over that of animals on fat-free or low-fat diets, as reported by Scheer et al. ('47), was also confirmed. In addition, we demonstrated that the iodine number of body lipids of the fat-deficient rats is lower than that of the rats on 25% fat diets.

TABLE 6

Metabolic water formed by rats fed various diets [1]

DIET	ML/100 GM RAT/DAY			
	Total	Fat	Protein	Carbohydrate
Fat-free	4.53	...	1.14	3.39
5% lard	4.44	0.49	1.12	2.83
25% lard	4.33	1.95	0.90	1.48
5% synthetic triglyceride	4.13	0.45	1.05	2.63
25% synthetic triglyceride	4.31	1.95	0.89	1.47

[1] Assuming oxidation of 100 gm of fat, protein and carbohydrate yields 107, 41 and 56 ml of water, respectively.

The higher urinary and fecal nitrogen excretion of the fat-deficient rats is probably due to (1) greater food consumption resulting in a higher nitrogen intake, and (2) inefficiency in utilization of ingested protein due to lack of fat. With high fat content in the diet, on the other hand, caloric requirement can be met with less food consumption. Furthermore, in the presence of an adequate amount of fat in the diet, the ingested protein is more efficiently utilized. Thus the relatively constant protein composition of tissues is maintained. This belief can be further strengthened by the fact that the percentage of nitrogen retention of the rats fed 25% fat diets was higher than that of the fat-free group (table 3).

The synthetic triglyceride preparation is well absorbed by rats as indicated by the high percentage of retention.

SUMMARY

The nutritional value of a synthetic triglyceride preparation composed of oleic acid, 60.0; palmitic acid, 30.0; stearic acid, 7.4; and linoleic acid, 2.6% was studied using weanling male rats of the Sprague-Dawley strain as experimental subjects. Rats of the same age, sex, and strain fed fat-free and lard-containing diets were used as controls. Weights of rats on various diets were recorded weekly; food intake, water and nitrogen balances were measured and fecal fat was determined. Body composition was analyzed following sacrifice at the end of the 13-week period of experimentation.

It was found that the synthetic triglyceride preparation incorporated in the diet at 5 and 25% levels was biologically utilized in the body, using weight gain, food intake and nitrogen and water balances as criteria. The average daily gain in weight was 2.49 gm per rat for the fat-free group whereas rats fed 5% lard, 25% lard, 5% synthetic triglyceride, and 25% triglyceride diets gained 3.18, 3.45, 2.91, and 3.27 gm per rat per day, respectively. However, the rats on fat-free diet consumed a greater amount of food with a higher caloric intake. The water intake of rats fed the fat-free diet was greatly increased and the percentage of ingested protein retained was decreased. The percentage of ingested synthetic triglyceride retained was from 94.6 to 98.6.

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CONCERNING THE ALLEGED OCCURRENCE OF AN "ANIMAL PROTEIN FACTOR" REQUIRED FOR THE SURVIVAL OF YOUNG RATS [1]

I. STUDIES WITH UNPURIFIED RATIONS

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In previous experiments reported from this laboratory (Schultze, '53a), a very high preweaning mortality was observed among young born to rats fed rations containing certain specimens of highly purified soybean protein. The mortality often reached close to 100% in the second or third litters born to the same mother, particularly if she was a survivor from a litter whose dam had received the same ration. Crude soybean oil meal, "vitamin-free casein," and a crude animal protein preparation made from defatted brain and spinal cord had a marked curative effect on this condition, as did, for unknown reasons, certain specimens of purified soybean protein (Schultze, '53b). Moruzzi, Piccioni and Rabbi and their associates (Piccioni et al., '50, '51; Moruzzi et al., '51; Rabbi et al., '51) described in several papers a similar high mortality of young rats which they produced by feeding to the mothers a ration containing 88% of a mixture of equal parts of ground wheat, maize, barley, oats and rye supplemented with wheat germ, calcium lactate, sodium chloride and 5% of casein which had been exhaustively washed with 0.05 N acetic acid (Rossi and Piccioni, '50); in addition the rats were fed fresh vegetables and dry yeast twice a week.

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Among F_1 generation young from mothers fed this ration the preweaning mortality was about 70%; in the F_2 generation it rose to 100%. The poor reproductive performance and survival of the rats could be aggravated by the addition of 0.6% of sulfaguanidine to the ration (Rabbi et al., '50). However, if crude casein were substitued for washed casein, the survival of young was usually close to 100% (Piccioni, '50). The Italian investigators considered the deficient ration to be lacking in a water-soluble, heat-stable "animal protein factor" associated with crude casein (Piccioni et al., '50), not identifiable with any of the known B vitamins, including vitamin B_{12} (Moruzzi et al., '51). Histological abnormalities in the liver (Piccioni et al., '51), kidneys, spleen and skeletal muscles of the newborn were observed (Rabbi et al., '51). More recently, they have reported the occurrence of alopecia and of neoplastic growths in older animals fed the same ration (Rabbi et al., '53). The high incidence of mortality could be reduced by adding 0.5% of choline to the ration (Rabbi and Piccioni, '52) and the skin lesions were cured by feeding a heat-inactivated culture of *Escherichia coli* (Piccioni et al., '53).

Many trials involving large numbers of animals from several successive generations have been made in this laboratory with the soybean protein ration (Schultze, '53a and b). However, the very high incidence of death of the young was not obtained with sufficient consistency to warrant, with this basal ration, a search for very potent sources of a curative factor and attempts to concentrate it.

Useful information concerning the possible existence of unknown dietary factors associated with some proteins might, however, be obtained by other approaches to the problem. One, involving the use of protein-free amino acid-containing rations, is reported in another paper (Schultze, '55a). The other approach, summarized herewith, is based on the experience of the Italian investigators and involves the use of modifications of their ration. The two principal modifications are reported here as experiments I and II. These

experiments have been in progress during the last three years. They were initiated independently but are reported jointly because they deal with the same general problem.

EXPERIMENTAL

Rations. The components of the rations used are listed in table 1. The rations for experiment I contained mainly ground, mixed cereals and casein and they are designated as MCC. They were similar to those used by the Italian investigators but contained added B vitamins, fat-soluble vitamins, choline, corn oil and a salt mixture. The protein content was 15 to 16% depending on the source of the cereals. When it became apparent that the growth of rats with these rations was subnormal, the rations used for experiment II were devised. These contained mainly rolled oats and casein and they are designated as OC. They provide a higher protein intake (21% on the basis of nitrogen analysis) of higher biological value (Block and Mitchell, '46) than the MCC rations. In addition, the rations contained a complete mixture of the B vitamins, a salt mixture and 2% of wheat germ oil to assure a good source of the unknown fat-soluble factor which Keane et al. ('51) reported to be necessary for the survival of young rats. Fresh vegetables and yeast were not used in these experiments.

Special attention was given to the preparation of casein which, on the basis of the observations of Piccioni et al. ('50) would appear to be the sole source, in the rations used, of the factor preventing the mortality of the young. The casein was prepared batchwise from fresh cows' skimmilk by 4 different procedures to yield:

Casein I (crude): The casein was precipitated at room temperature by the addition of 10% acetic acid to pH 4.6. The supernatant was siphoned off after settling of the curd; the latter was strained through cheesecloth and washed twice with dilute acetic acid pH 4.6 equal to 2 volumes of the original milk. The curd was finally strained, squeezed dry, dried at about 70°C. and ground.

Casein II: The initial precipitation of the casein was carried out as described above. The separated curd was then leached over a period of two weeks with a total of about 70 changes of 0.5% acetic acid, using each time about 10 l per kilogram of moist curd. Toluene was used as a preservative.

TABLE 1

Composition of rations

COMPONENTS	DESIGNATION OF RATION			
	MCC	OC_1	OC_2	OC_3
	gm	*gm*	*gm*	*gm*
Ground mixture of equal parts wheat, oats, barley, maize, rye	825.0	...	...	...
Rolled oats, ground in hammermill	...	840.0	840.0	840.0
Casein I and/or II (see table 2)	50.0	...	...	...
Casein III, leached at pH 4.6	...	65.0	65.0	...
Casein IV, leached at pH 3.6	...	...	...	65.0
DL-methionine	...	3.3	3.3	3.3
Sucrose + vitamin mixture I [1]	...	20.0	20.0	20.0
Sucrose + vitamin mixture II [2]	20.0	...	...	...
Corn oil + vitamins A, D, E [3]	50.0	...	...	...
Corn oil + vitamins A, D [4]	...	10.0	10.0	10.0
Wheat germ oil	...	20.0	20.0	20.0
Salts IV [5]	40.0	30.0	30.0	30.0
Calcium lactate	15.0	...	...	...
Sucrose	...	11.7	1.7	11.7
Condensed whey solubles [6]	...	...	10.0	...
Total	1000.0	1000.0	1000.0	1000.0

[1] 20 gm of the mixture contain the following compounds to furnish per kilogram of mixed ration: 5 mg thiamine chloride; 10 mg riboflavin; 5 mg pyridoxine hydrochloride; 50 mg calcium pantothenate; 20 mg nicotinic acid; 400 mg i-inositol; 1000 mg choline chloride; 0.2 mg folic acid; 10 mg para-aminobenzoic acid; 0.2 mg biotin; 0.04 mg vitamin B_{12}; 5 mg 2-methyl-1,4-naphthoquinone.

[2] 20 gm of the mixture contain the following compounds to furnish per kilogram of mixed ration: 15 mg thiamine chloride; 30 mg riboflavin; 50 mg pyridoxine hydrochloride; 50 mg calcium pantothenate; 100 mg nicotinic acid; 100 mg i-inositol; 500 mg choline chloride; 2 mg folic acid; 200 mg para-aminobenzoic acid; 1 mg biotin; 5 mg 2-methyl-1,4-naphthoquinone.

[3] 50 gm of the mixture contain 250 mg d,l-α-tocopherol; and 75,000 Int. Units vitamin A plus 1,250 Int. Units vitamin D as furnished by Haliver oil.

[4] 10 gm of mixture contain 10,000 Int. Units vitamin A acetate; 1,500 Int. Units vitamin D_3.

[5] Schultze, '50a.

[6] The Borden Co.

The curd was finally collected in cheesecloth, squeezed dry, dried at 70°C. and ground.

Casein III: Forty gallons of skimmilk were diluted with two volumes of water and warmed to 34.5°C. in a jacketed, stainless steel vat. The casein was precipitated by the addition of dilute hydrochloric acid to pH 4.6 with gentle stirring (Palmer, '26). The whey was drained and the curd leached during a 14-day period with 40 changes of 60 l each of water adjusted to pH 4.6 with acetic acid. Toluene was used as the preservative. Between washings, the suspended casein was vigorously stirred and the liquid was siphoned off as completely as possible after settling of the precipitate. The resulting product was finally washed with two changes of distilled water, partially dehydrated with 95% ethanol, dried at 70% and ground in a hammermill.

Casein IV: The procedure was the same as described for Casein III except that the curd was leached with 40 changes of 60 l each of 0.05 N acetic acid which yielded a pH of 3.6.

Animals. For experiment I, 30 females and 10 males, about 4 weeks old, from a hooded strain of our stock colony were fed ration MCC_1 (see table 2) which contained 5% of the crude casein I. They were mated when the females weighed about 150 gm and their young (F_1 generation) were allotted to each of the 4 rations shown in table 2. When the females weighed about 150 gm, they were mated and their young (F_2 generation) were in turn fed the same ration as their parents. These animals were also mated when they weighed about 150 gm and their offspring (F_3 generation) were raised through a 6-weeks' growing period.

The rats used for experiment II were of the black strain of Line 3 maintained in this laboratory for many years. Their ancestors had been fed rations devoid of animal protein for several generations (Schultze, '53a). The females of the parent (P) generation had been fed, since weaning and through the first lactation, a ration containing purified soybean protein. They were then transferred to ration OC_1. Their subsequent litters (F_1 generation) and two generations

TABLE 2

Reproductive performance of rats fed mixed cereal-casein rations (experiment I)

	RATION			
	MCC_1	MCC_2	MCC_3	MCC_4
TYPE OF CASEIN	5% I (crude)	2.5% I (crude) 2.5% II (leached)	5% II (leached)	5% II (leached)
ADDED VITAMIN B_{12}	None	None	None	40 μg per kg
		F_1 generation		
Number of pregnancies	8 (8)[1]	8 (8)	27 (8)	21 (8)
Number of litters born alive	8	8	27	21
Number of litters weaned	8	8	21	19
Number of living young per litter	7.4	8.1	7.7	6.8
Mortality of young in 30 days, %	18.0	27.3	50.8	34.7
Mean weight of young at 30 days (gm)	42.0	41.8	40.7	39.9
Mean 6-weeks' weight gain of young[2] (gm)	94.7	91.2	43.7	69.1
		F_2 generation		
Number of pregnancies	15 (15)	13 (13)	33 (20)	29 (21)
Number of litters born alive	15	13	32	29
Number of litters weaned	15	13	0	19
Number of living young per litter	8.2	7.6	7.8	7.3
Mortality of young in 30 days, %	8.7	21.3	100	68.7
Mean weight of young at 30 days (gm)	38.7	35.4	..[3]	26.8
Mean 6-weeks' weight gain of young[2] (gm)	87.0	83.6	..	53.0

[1] Number of females in parentheses.

[2] From 30 to 72 days of age.

[3] All young died within 10 days after birth.

of offspring of the latter (F_2 and F_3 generations) were used for the experiments summarized here. Except shortly before parturition and during lactation, the rats were housed in groups of 4 or 5 in cages provided with raised screen bottoms. When the females were 10 weeks old, they were bred with males fed the same ration. Shortly before parturition, the pregnant animals were housed singly in cages bedded with clean wood shavings. The size of the litter was not reduced regardless of the number of the young born. The young were weighed daily and they were weaned at three weeks of age. After weaning or loss of a litter, the females were placed with males again immediately. For comparison of ration OC_1 with OC_2 or OC_3 respectively, matched littermate females were selected and fed the respective rations from the time they were weaned. The experiment was continued until females of three filial generations had produced 4 litters each.

RESULTS AND DISCUSSION

Experiment I. All of the females of the parent generation became pregnant, cast live litters (mean of 7.4 living young per litter) and raised one or more young to 30 days of age with a mean weight of 45.7 gm. The total mortality of these young was 11.3%. Duing the 6 weeks' postweaning period, the young females of the F_1 generation gained an average of 117.3 gm. The reproductive performance of the F_1 and F_2 generations fed 4 different modifications of the MCC rations is summarized in table 2. In both the F_1 and F_2 generations, the rats receiving the ration containing 5% of crude casein I continued to have a satisfactory reproductive performance and a low mortality of the young. The mean weight of the young at 30 days of age and their postweaning weight increments in 6 weeks, however, were below those normally observed with this strain. When 2.5% of crude casein was replaced by an equal amount of leached casein II, the mortality of the young increased somewhat in both the F_1 and F_2 generations. When the ration contained only the leached casein II, the mortality of the young from the F_1 generation females increased to about 50% but

all of the young cast by F_2 generation females died within 10 days after birth. This condition could be partly corrected by the addition of vitamin B_{12} to the maternal ration in both the F_1 and F_2 generations. However, the preweaning and postweaning weight gains of the survivors were very inferior.

Visual inspection of the young of all groups indicated that during the first two or three days of life they received an abundance of milk. On subsequent days, however, many of the young of mothers fed rations MCC_3 or MCC_4 did not appear to have any milk in their stomachs and they usually died within two days.

With the females fed rations MCC_3 or MCC_4 considerable difficulty was experienced in obtaining pregnancies in spite of frequent exchange of different fertile males. There was no evidence, however, of late resorption of whole litters (as revealed by weight changes) and no abortions or maternal deaths at the time of parturition occurred. Alopecia or neoplastic growths were not observed in these animals.

With respect to the survival of the young, this experiment confirms the observations of the Italian investigators although it should be noted that the 100% mortality was not obtained until the F_3 generation of young was reached. The procedure adopted for purification of casein II yielded a product which, as a supplement to a vitamin B_{12}-deficient mixed cereal grain ration, was no longer able to support the survival of the rats through several generations. Addition of vitamin B_{12} to this ration permitted the survival of only about one-third of F_3-generation young.

Experiment II. The OC rations described in table 1 supported rapid weight gains of the young (for instance, 120.1 ± 1.26 gm for 80 females and 189.7 ± 7.8 gm for 22 males during the 6 weeks' postweaning period) and a high rate of fertility in mature animals throughout three filial generations. No indications of alopecia or other abnormalities not associated with reproduction were observed. The general appearance and health of the animals seemed to be excellent after they were weaned. The principal observations concerning the re-

productive performance of these animals and the survival of their young are summarized in table 3.

Although in some groups, particularly 7 and 8, the preweaning mortality of the young was high, it never occurred to the extent reported for the offspring of the F_1 generation by Moruzzi and his associates or as recorded in experiment I of this paper for the offspring of the F_2 generation fed the vitamin B_{12}-deficient ration. There was no consistent increase in the mortality of the young with successive generations and the results were about the same whether casein was leached at its iso-electric point or with 0.05 N acetic acid at pH 3.6 (compare groups 5 and 7 with groups 6 and 8 respectively). While litters of offspring of rats fed casein IV, leached at pH 3.6, showed the highest mortality (groups 7 and 8), the fact that the first litters of group 8 had a mortality of only 23.7% emphasizes that this method of preparing casein, under the conditions of experiment II, gave a product which could not be used to demonstrate the existence of an unknown dietary essential associated with casein. The addition of a whey concentrate to the ration decreased the mortality of the young somewhat but it did not improve the reproduction or lactation (compare groups 1 and 2).

The number of young born dead, excluding abortions of whole litters, did not exceed about 2% in any group and most of the post natal deaths occurred between the 8th and the 15th day post partum. The reason for the deaths is not known. With few exceptions, the amount of milk secreted by the mothers appeared to be abundant. Data presented in an earlier paper (Schultze, '54) demonstrated that the rolled oats-casein ration could support large weight increments of litters containing up to 14 suckling young. The 21-day weight attained by the young in this experiment was about the same in all groups. It approached that attained in 30 days by the young of mothers fed the MCC_1 ration. In most instances, the mothers gained weight during the lactation period. Throughout experiment II, abortions near term occurred with a relatively

TABLE 3

Reproductive performance of rats fed oats-casein rations

GROUP	LITTER SEQUENCE	NUMBER OF					MORTALITY IN 21 DAYS	MEAN 21 DAY WEIGHT OF YOUNG	MEAN WEIGHT CHANGE OF MOTHER [1]
		Pregnancies	Abortions (A) Resorptions (R) Maternal deaths (D)	Litters born alive	Litters weaned	Living young per litter			
							%	*gm*	*gm*
1 [2]				Ration OC_1, F_1 generation					
	1st	23	2A	21	21	6.90	15.2	38.0	+ 13.8
	2nd	22	1A	21	19	9.81	54.8	38.3	+ 14.4
	3rd	21	1R	20	17	10.00	55.1	39.8	+ 3.3
	4th	20	1A;1R	18	17	9.28	29.3	36.1	— 6.2
2 [2]				Ration OC_2, F_1 generation					
	1st	21	0	21	21	8.19	17.4	38.2	+ 25.1
	2nd	20	1A	20	17	9.32	31.6	37.2	+ 12.4
	3rd	20	1A	20	17	9.85	32.0	36.6	— 0.4
	4th	19	1A	19	17	9.11	18.5	36.1	— 5.9
3				Ration OC_1, F_2 generation, offspring from group 1					
	1st	50	1A	49	43	7.94	33.3	36.5	+ 16.0
	2nd	50	4A;1R	45	43	8.28	24.3	37.4	+ 9.8
	3rd	50	7A;2R	41	36	8.51	20.8	37.7	+ 10.5
	4th	50	10A	40	38	9.03	34.1	39.2	+ 11.8
4				Ration OC_1, F_3 generation, offspring from group 3					
	1st	26	1A	25	24	7.96	13.7	36.8	+ 19.2
	2nd	25	3A	22	22	8.64	25.0	35.9	+ 14.3
	3rd	22	0	22	18	8.73	41.1	37.0	— 11.1
	4th	22	3A	19	10	9.00	59.6	36.1	— 11.7

5 [3]			Ration OC_1, F_2 generation, offspring from group 1						
	1st	19	0	19	17	6.68	7.07	40.4	+ 10.4
	2nd	19	1A	18	17	8.83	24.3	37.0	+ 14.6
	3rd	19	2A	17	17	8.82	16.8	37.8	+ 12.5
	4th	19	3A	16	12	7.25	54.3	40.0	+ 3.5
6 [3]			Ration OC_3, F_2 generation, offspring from group 1						
	1st	21	1R	20	18	7.05	16.3	40.6	+ 15.0
	2nd	21	1A	20	17	7.35	15.7	37.6	+ 21.6
	3rd	21	1A	20	18	8.50	22.9	37.1	+ 21.1
	4th	20	1A	19	15	6.84	40.4	39.2	+ 9.8
7 [4]			Ration OC_1, F_3 generation, offspring from group 6						
	1st	21	0	21	14	8.29	57.5	40.3	+ 25.6
	2nd	20	0	20	10	8.40	75.0	37.0	+ 10.4
	3rd	19	3D	16	6	9.37	81.3	35.3	+ 3.5
	4th	16	1A	15	9	8.27	61.3	38.9	+ 8.7
8 [4]			Ration OC_3, F_3 generation, offspring from group 6						
	1st	21	1A	20	18	8.80	23.7	38.7	+ 26.0
	2nd	21	2A	19	13	8.26	50.3	35.0	+ 12.1
	3rd	20	3A	17	9	9.11	71.0	34.5	+ 9.2
	4th	18	3A	15	11	8.60	61.2	37.3	+ 6.5

[1] During lactation.
[2] Matched littermates; offspring from parents fed ration OC_1.
[3] Matched littermates.
[4] Matched littermates.

high incidence of 7%. This condition did not always occur in successive pregnancies of the same rats.

Because the present experiments were extended over two to three generations, the maternal carry-over of compounds deficient in the ration should have been at a minimum. Furthermore, in other experiments (Schultze, '55b), we have reared rats on protein-free amino acid rations; their offspring, while receiving ration OC_1 since weaning, produced litters which had a high rate of survival. Whether or not synthetic activities of the microflora in the alimentary tract of our animals account for the failure to produce in experiment II a consistently high mortality of the young cannot be decided at the present time. However, in an earlier study with the same strain of rats the inclusion of 2% of phthalylsulfathiazole in a vitamin B_{12} ration devoid of animal protein improved the reproductive performance of rats and decreased the mortality of the young (Schultze, '50b).

The different results obtained in these two experiments clearly demonstrate that specific conditions — as yet only empirically defined—are required to produce a high preweaning mortality in rats. While the rations used contained different ingredients, they are similar with respect to the alleged "animal protein factor" since extensively leached casein was used in both experiments. Whether the slight difference in initial precipitation (hydrochloric acid versus acetic acid) of the casein, or the higher level at which it was used in experiment II could account for the difference in results cannot be decided at present. Other components, not of animal origin, such as the methionine, the higher level of choline, the protein of higher biological value and the wheat germ oil, singly or jointly, together with casein III or IV yielded a ration with which the need for an unknown "animal protein factor" could not be demonstrated.

This experience emphasizes the need for studies on the dietary requirements for reproduction and lactation with rations of which all components are pure compounds of known structure. Until results of such experiments are at hand, it

appears premature to interpret the results presented by the Italian investigators and in experiment I of this study as constituting conclusive evidence for the existence of an unknown "animal protein factor" required for the survival of young rats. The results of our studies with protein-free amino acid rations, reported in another paper, support this view. Furthermore, it may be recalled that it has been possible to maintain rats through 10 filial generations with rations devoid of animal protein (Schultze, '53a).

SUMMARY

1. For a study of the alleged occurrence of an unrecognized "animal protein factor" required for survival of young rats, the usefulness of rations containing exhaustively leached casein plus a mixture of cereal grains or rolled oats was investigated.

2. When casein was leached for two weeks with 0.5% acetic acid and fed at a 5% level as a component of a vitamin B_{12}-deficient ration containing 82.5% of a mixture of cereal grains, there was an increase in mortality of young rats in successive generations until it reached 100% in the F_3 generation young. Vitamin B_{12} reduced mortality to about 67%.

3. When similar casein preparations, leached at pH 4.6 or 3.6 were fed at a 6.5% level as components of rations containing 84% rolled oats and other ingredients of synthetic or plant origin, a high preweaning mortality of young rats was not consistently observed through 4 successive generations.

4. These experiments emphasize that specific conditions, as yet only empirically defined, are neccessary for establishment of a high preweaning mortality of the young.

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CONCERNING THE ALLEGED OCCURRENCE OF AN "ANIMAL PROTEIN FACTOR" REQUIRED FOR THE SURVIVAL OF YOUNG RATS

II. REPRODUCTION OF RATS FED PROTEIN-FREE AMINO ACID RATIONS [1]

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Earlier experiments reported from this laboratory (Schultze, '53a) demonstrated that the feeding of rations containing certain specimens of purified soybean proteins to rats for several generations produced a high incidence of deaths among the young. This was presumably due to lactation failure. Soybean oil meal, "vitamin-free" casein and a crude animal protein preparation made from defatted brain and spinal cord each had a marked curative effect. Piccioni et al. ('51) interpreted their experiments with cereal grain rations as demonstrating the existence of an unknown "animal protein factor" required for the survival of young rats. Gander and Schultze ('55), while confirming some of the observations of Piccioni et al. under certain specific conditions, concluded that these experiments did not constitute decisive evidence for the occurrence of an unrecognized nutrient of animal origin. If such a compound essential for lactation and for survival of the young were associated with proteins, a deficiency in this nutrient might best be established with protein-free rations containing amino acids, and perhaps ammonium salts, as the only source of dietary nitrogen (aside

[1] Paper no. 3240, Scientific Journal Series, Minnesota Agricultural Experiment Station.

from the small amount in the pure vitamins and the lipid components of the ration). Studies with such rations would have further interest because they would constitute a closer approach to the goal of preparing, from compounds of known chemical structure, a ration which is adequate to support growth and reproduction of mammals for several generations.

No detailed information appears to have been published concerning the reproductive performance of mammals fed amino acid rations. Rose ('47), referring to unpublished work in his laboratory with 10 "essential amino acids," stated that "upon such mixtures, rats not only grew quite satisfactorily but are able to reproduce and rear their young." According to an abstract of a paper presented by Emerson et al. ('47), young rats born to mothers fed a mixture of 10 amino acids in lieu of protein had subnormal preweaning and postweaning growth rates. Vitamin B_{12}, which became available later, did not overcome this retardation of growth.[2] No quantitative information was given concerning the reproductive performance of the female rats.

This paper summarizes attempts to rear rats through more than one generation with rations of which as much as 97% consisted of compounds of known chemical structure.

EXPERIMENTAL

Rations. Amino acid mixture I was patterned after that of Rose and Smith ('50), with which, when it was supplemented with ammonium citrate, they had observed fairly good postweaning weight gains of the young rats obtained from a stock colony. Shelton et al. ('50) fed this mixture successfully to pigs. The amino acid mixture II was that which Schweigert and Guthneck ('53) had used successfully for the determination of the availability of lysine in foods. The composition of the amino acid mixtures is shown in table 1 and that of the principal rations used in table 2. Wheat germ oil was included in the rations as a source of the unknown factor

[2] Personal communication from Dr. G. A. Emerson.

which Keane et al. ('51) have reported to be necessary for the survival of young rats.

The performance of rats with respect to growth and reproduction was in many instances compared with that of rats of the same genetic background reared simultaneously, for different purposes, on a ration containing 84% ground rolled oats, 6.5% leached casein and the same mixtures of added

TABLE 1

Composition of amino acid mixtures

COMPONENT	SOURCE	MIXTURE I	MIXTURE II
		gm	*gm*
L-Arginine monohydrochloride	(1)	2.50	5.10
L-Histidine monohydrochloride	(2)	5.00	3.47
DL-Isoleucine (Lots 101772; 103331)	(2)	10.00	13.30
L-Leucine	(3)	8.00	12.65
L-Lysine hydrochloride (95%)	(4)	13.18	7.37
DL-Methionine	(5)	6.00	3.60
DL-Phenylalanine	(2)	9.00	5.33
DL-Threonine	(2)	10.00	8.00
DL-Tryptophan	(2)	2.00	1.85
DL-Valine	(2)	14.00	14.40
DL-Alanine	(2)	...	5.77
DL-Aspartic acid	(2)	...	6.48
L-Cystine	(2)	...	3.34
L-Glutamic acid	(2)	...	24.50
L-Tyrosine	(3)	...	6.60
Glycine	(2)	...	0.51
Total		79.68	122.67

(1) H. M. Chemical Co., Ltd.; (2) Mann Research Laboratories; (3) Staley Mfg. Co.; (4) Du Pont de Nemours and Co.; (5) U. S. Industrial Chemicals Co.

salts, lipids and vitamins as were used in the amino acid rations (Schultze, '54). The nitrogen content of this ration was 3.34%; the nitrogen contributed by the amino acids and ammonium citrate in the amino acid rations AA_2, AA_3 and AA_5 was 1.62%; in the amino acid ration AA_4, 2.60%; and in the amino acid ration AA_{11}, 1.48%.

During the course of these experiments, it became apparent that the growth rate of all rats fed rations AA_2, AA_3 and AA_5

was much lower than that which Rose and Smith ('50) had observed with essentially the same amino acid and ammonium citrate mixture. Tests with a few rats indicated that when these rations were modified by the addition of 0.4% of liver extract [3] or with three times the amount of B vitamins or with 0.92% of sodium bicarbonate an increased growth rate was not obtained. The substitution of 64.9% of dextrinized

TABLE 2

Composition of rations

COMPONENT	RATION NO.		
	AA_3	AA_4	AA_{11}
Amino acid mixture I, gm	79.68	159.36	....
Amino acid mixture II, gm	....	....	122.27
Diammonium citrate, gm	51.65	51.65	....
Salt mixture IV,[1] gm	40.00	40.00	40.00
Sucrose + B vitamin mixture,[1] gm	20.00	20.00	20.00
Sucrose, gm	778.67	698.99	787.73
Wheat germ oil, gm	20.00	20.00	20.00
Corn oil + vitamins A and D_3,[2] gm	10.00	10.00	10.00
Pyridoxine hydrochloride, mg	5	5	....
Vitamin B_{12}, μg	40	40	40
Total, gm	1000.00	1000.00	1000.00

[1] See Schultze, '53b.

[2] Ten grams corn oil contained 10,000 Int. units vitamin A acetate and 1,250 Int. units vitamin D_3.

Note: In ration AA_2, 50 gm of hydrogenated vegetable oil (Crisco) replaced 50 gm of sucrose of ration AA_3. In ration AA_5, 648.67 gm of dextrinized cornstarch replaced 648.67 gm of sucrose of ration AA_3.

cornstarch for an equal weight of sucrose produced only a small increase in growth rate. Chromatographic examination of the amino acids used in mixture I revealed that only DL-isoleucine contained a contaminant which reacted with ninhydrin. This contaminant comprised about 26% of the DL-isoleucine used. It had an R_f value in l-butanol-acetic acid-water (4:1:5 v/v) of 0.63 compared to 0.59 found for the prin-

[3] Liver Concentrate N. F., The Wilson Laboratories.

cipal component and for other specimens of DL-isoleucine [4] previously obtained from the same source. When the amount of DL-isoleucine (lot 103331) in ration AA_3 was increased by 30%, improved but still subnormal growth was obtained in a small trial involving 5 rats. Therefore, rations AA_2, AA_3 and AA_5, when judged by the recommendations of Rose et al. ('49) were partially deficient in isoleucine. The amino acid mixture II used in ration AA_{11} was compounded with remaining supplies of another specimen of DL-isoleucine, lot 100027,[4]

TABLE 3

Weight gains of rats fed amino acid rations

RATION	RATS			WEIGHT AT WEANING	WEIGHT GAIN IN	
	Sex	No.	Generation		6 weeks	12 weeks
				gm	*gm*	*gm*
AA_2 or AA_3	F	22	P	43.8 ± 1.04 [1]	52.1 ± 2.38 [1]	105.3 ± 2.51 [1]
AA_{11}	F	5	P	43.5	97.0	. .
AA_3	F	9	F_1	19.9 ± 2.22	44.0 ± 2.55	96.4 (5) [2]
AA_3	M	7	F_1	20.0 ± 1.60	44.0 ± 1.72	96.8 (4) [2]
AA_4	M	10	F_1	23.4 ± 2.64	100.7 ± 5.16	134.7 (3) [2]
AA_4	F	13	F_1	20.0 ± 1.60	89.7 ± 4.25	. .

[1] Standard error of the mean.

[2] Number of rats carried to 12 weeks, in parentheses.

and later (during the second pregnancy and lactation) with a specimen from a different source [5] which produced only one spot when tested chromatographically.

Animals. All rats were from the black strain of Line 3 which had been used in our earlier studies referred to above. Those designated as the parent (P) generation were fed the amino acid rations from the time they were weaned at three weeks of age, except in the instance of 6 multigravid mature females (group 1, table 4) who had completed previous pregnancies while fed the oats-casein ration. Breeding of young

[4] Lot 100027, Mann Research Laboratories.

[5] Merck and Co., stated by the manufacturers to consist of L-isoleucine 50% with D-alloisoleucine.

females started when they weighed 140 to 150 gm. They were kept in groups of 4 to 5 in cages with raised screen bottoms. A few days before parturition the females were transferred to individual cages bedded with clean wood shavings. Close inspection of the cages did not reveal fragments of fecal pellets suggesting that coprophagy was not practiced extensively by these animals. The males used, in most instances, were mature animals that had been fed the rolled oats-casein ration. The offspring (F_1 and F_2 generation) of the mothers fed amino acid rations were not weaned until they were 28 days old, due to their small size.

RESULTS AND DISCUSSION

Growth. While these studies were not primarily concerned with the growth-promoting properties of the ration, the weight increment of young rats furnished pertinent information on the adequacy of the rations used. The data summarized in table 3 indicated that even when the "essential amino acids" were furnished at the 16% level as in ration AA_4 which contained additional nitrogen in the form of ammonium citrate, the growth rate of the rats was inferior to that obtained with this strain of animals fed natural rations. (With the rolled oats-casein ration, for instance, the 6-week postweaning weight increments were 120.1 ± 1.26 gm for 80 females and 189.7 ± 7.8 gm for 22 males.) Some other investigators (Ramasarma et al., '49) have also observed inferior growth with rations containing only the "essential amino acids." No difference was noted in this respect between rations AA_2 and AA_3. The substitution of dextrinized cornstarch (ration AA_5) for most of the sucrose in ration AA_3 appeared to improve the ration somewhat. Womack et al. ('53) have observed a favorable effect of dextrinized cornstarch on the nitrogen balance of rats fed low levels of the amino acid rations. Several investigators reported a favorable effect of cornstarch on growth of rats or chickens (see Harper and Katayama, '53). The growth rate of the rats of the F_1 gen-

eration, because of early malnutrition is reduced even when they are fed an adequate ration (Schultze, '55).

Reproductive performance. While no systematic study of their reproductive performance was made, mature males transferred from the rolled oats-casein ration to the amino acid rations maintained their fertility for at least 5 to 6 months. Among young males of the F_1 generation fed the AA_4 or AA_5 ration, several were able to breed successfully. Among those F_1 generation males that weighed only about 20 gm at 4 weeks of age, several developed a persistent penile prolapse. Table 4 is a summary of the reproductive performance of females including survival and preweaning weight gains of their young. With females of the P generation fed amino acid mixture I, the interval between exposure to fertile males and the first conception which led to a completed pregnancy was much longer (mean of 30.5 days for 20 rats of groups 2 and 4, table 4) than is usually observed with animals of the same strain fed the rolled oats-casein ration (mean of 11.2 days for 50 rats from parents fed the amino acid rations). This delay could be due to early embryonic death of the whole litter as observed by Nelson and Evans ('53) under certain conditions of feeding protein-free rations for short intervals. Other factors which may have contributed to the long interval between breeding and production of litters may be an abnormally long estrous cycle, defective ova or inadequate fertilization and implantation of the ova. In group 8, fed amino acid mixture II, the interval referred to was only 16.5 days. From 76 females exposed to fertile males, 6 showed no indication of pregnancy within 4 months. Among 68 pregnancies of rats fed the amino acid rations containing sucrose plus 8% of amino acid mixture I, there occurred 11 maternal deaths shortly before or immediately after parturition. In contrast to this, among 51 pregnancies of the P, the F_1 and F_2 generations fed the same mixture at a 16% level there were only two maternal deaths (including one in the F_2 generation not shown in table 4). Among groups 1 to 4 and 6 and 7, the weight increments of the mothers during preg-

TABLE 4

Reproductive performance of rats fed amino acid rations

	GROUP							
	1	2	3	4	5	6	7	8
Ration	AA_3	AA_3	AA_3	AA_2	AA_4	AA_4	AA_5	AA_{11}
Generation	P [1]	P [2]	F_1	P [2]	P [2]	F_1	P [2] + F_1 [3]	P [2]
Number of mothers	6	12	7	11	2	14	4	5
Number of pregnancies	6	25	11 [4]	26	7	28	12	10
Mean weight gain during pregnancy, gm	37.5	30.5	30.9	37.2	64.4	41.1	42.5	67.7
Number of maternal deaths near parturition	1 [5]	6	3	1	0	1 [5]	0	0
Number of litters born	6	19	6	25	7	27	12	10
Total number of young born	36	101	24	151	49	125	66	76
Number of young born dead	9	2	2 [6]	20 [7]	0	3	0	0
Number of survivors, 4th day post partum	21	60	14	109	45	93	36	73
Mortality of young in 28 days, %	51.8	61.6	50.0	39.8	10.2	25.4	62.1	4.1
Mean weight of young at birth, gm	4.7	4.3	4.4	4.1	4.8	4.7	4.4	5.4
Mean weight of young, 21 days old, gm	15.1	15.7	18.6	15.3	24.0	22.2	16.7	19.1
Mean weight of young, 28 days old, gm	19.5	20.9	23.0	20.2	34.1	30.2	22.6	27.1
Mean weight change of mothers, 7 days, gm	— 32.8	— 9.0	— 1.1	— 12.4	— 9.1	— 7.4	— 11.7	— 20.0
Mean weight change of mothers, 28 days, gm	— 26.0	— 12.8	+ 3.0	— 11.5	— 7.0	— 12.0	— 13.4	— 24.4

[1] Mature rats fed diet AA_3.

[2] Amino acid rations were fed since weaning at 21 days of age.

[3] Two rats were offspring from group 4.

[4] Including 1 resorption.

[5] Died shortly after parturition.

[6] Including 1 abortion.

[7] Including 2 abortions.

nancy were subnormal. This is partly due to the relatively small number of young born per litter, which, in 114 litters obtained with the rations containing amino acid mixture I, averaged only 5.25. The number of young born per pregnancy decreased progressively from group 1 to group 3, the longer ration AA_3 was fed. In contrast to this, rats of the same strain fed the rolled oats-casein ration had an average of 8.08 (175 litters) and 8.96 (165 litters) young in the first and second pregnancies respectively. For the 10 litters obtained with the ration containing amino acid mixture II the average number of young born was 7.6. In groups 1 and 4, a relatively high proportion of the young were born dead.

Lactation. Among 114 litters born to the rats fed the amino acid mixture I, 8 died because 6 of the mothers among those in groups 2, 5 or 7 either had no milk or made no attempt to feed the young. Cannibalism caused the early loss of two litters of one mother in group 4. With these exceptions, there appeared to be an abundance of milk in the stomachs of all young during the first two days post partum. Later the amount of milk appeared to be less. The mean weight of the young, at 21 and 28 days of age, was greatly subnormal particularly in the litters from mothers fed the 8% amino acid ration (groups 1 to 4 and 7). Although some individuals in these litters weighed only 11 to 12 gm at 28 days of age, they were very active and appeared "normal" except for size. Their bodies, however, appeared to be longer than those of normal young rats of the same weight (see Stewart, '18). The mothers, while nursing their young, incurred a marked weight loss particularly during the first 7 days post partum. In contrast to this, rats of this strain when fed the rolled oats-casein ration produced young that weighed on the average about 38 gm at three weeks of age and the mothers usually gained from 10 to 15 gm while nursing their first litters (Schultze, '54).

When the amino acid content of the ration was increased from 8 to 16%, the weight of the young, both at 21 and 28 days of age, though still subnormal, was increased. The

mothers, however, still lost weight under these conditions. Although a mother might be nursing only 3 to 4 young, most, if not all, of the 12 potentially functional mammary glands were used by the young. It appears evident that one of the principal reasons for subnormal preweaning weight of the young is inadequate lactation. Whether this involves an insufficient quantity or an abnormal composition of the milk or both cannot be decided from the available evidence.

An attempt was made to determine whether prenatal injury to the young was responsible for their subnormal growth. With three successive litters from the same mother fed ration AA_2, about half of the young were transferred to a

TABLE 5

Effect of amino acid ration on lactation of rats

NUMBER OF NURSING MOTHERS	RATION OF NURSING MOTHERS			
	$\frac{OC_1}{3}$[1]		$\frac{AA_2}{3}$[2]	
Source of young rats	Own	Foster	Own	Foster
Number of young 1 hour after birth	15	14	14	14
Mean weight of young at birth, gm	5.1	4.1	4.2	5.1
Number of young surviving, 3 weeks	10	9	8	10
Mean weight of young at 3 weeks, gm	37.5	24.7	12.7	13.9

[1] Rolled oats-casein ration.

[2] Three successive pregnancies and lactations of the same animal.

mother fed the rolled oats-casein ration that had cast a litter at the same time and about half of her young were transferred to the mother fed the amino acid ration. The data shown in table 5 indicate that young born to a mother fed an adequate diet during pregnancy failed to grow normally when nursed by an animal fed the amino acid ration. This must be due to inadequate nutrition during the preweaning period. The young born to mothers fed the amino acid ration made much better weight gains when nursed by a mother fed the oats-casein ration, but their growth was still subnormal. This might be a reflection of their smaller birth weight or of a

functional impairment due to improper prenatal nutrition, or of both factors.

Mortality of the young. Except in groups 5, 6 and 8 (table 4), the mortality of the young was high. Most of the deaths occurred during the first 4 days postpartum. After the 10th day, there were very few deaths even though the animals grew very slowly. With the ration AA_4, which contained 16% of the amino acid mixture I, there was a relatively low mortality in those litters in which there were any survivors. Thus, in 20 litters from the F_1 generation mothers (group 6, table 4), the mortality among 105 young that were born alive was only 13.3%, whereas in 7 litters, with only 17 young, none survived. In several instances, mothers were able to rear a litter of the second or third pregnancy after having failed to do so in the first or second pregnancy. When the lipid content of the ration containing 8% amino acids was increased from 3 to 8%, the mortality of the young was less but their preweaning weight gains were not increased (compare groups 2 and 4, table 4).

The data summarized in table 4 show that an increase of the amino acid mixture from 8 to 16% decreased the mortality and improved lactation. This was also observed with 6 mothers from groups 2 and 3 that were transferred to ration AA_4 after they had cast 13 litters with 77 young of which 68 died before they were three weeks old; after transfer to ration AA_4 these mothers cast 12 litters with 48 young, 20 of which died before the 4th day post partum but 27, from 5 litters, were weaned at 28 days of age when they had a mean weight of 29.8 gm.

Of special interest are the results obtained with ration AA_{11} (table 4) which contained the 16 amino acids of mixture II in which the total nitrogen content and the concentration of the "essential amino acids" (with the exception af arginine, leucine and isoleucine) are less than contributed by the amino acid mixture I and ammonium citrate to rations AA_2, AA_3 and AA_5. With ration AA_{11} there was a 96% survival of 76 young in 10 litters of the P generation, but the preweaning

growth of the young was subnormal. While these results involve relatively few animals and do not cover the more rigid test of reproductive performance of filial generations, they suggest that amino acid mixture II may be superior to mixture I with respect to reproduction and lactation. To what extent any of the amino acids contribute to this effect will require detailed study. The relative amounts of leucine, isoleucine and arginine are higher in amino acid mixture II than in I.

The reason for the relatively poor reproductive performance and lactation of rats fed the amino acid rations is not clear at present. With rations AA_2, AA_3 and AA_5, a partial isoleucine deficiency, superimposed on a relatively low nitrogen intake is no doubt involved. A partial threonine deficiency has previously been shown to lead to great weight losses of nursing mothers and to subnormal weight gains of the young (Schultze, '53b). When the concentration of all amino acids of mixture I was doubled, the absolute isoleucine deficiency was alleviated and the nitrogen content of this ration (AA_4) became equivalent to about 15% protein ($N \times 6.25$). A comparison of the reproductive and lactation performance of rats fed protein-free diets containing only the "essential amino acids" with that on a ration containing the same quantity of nitrogen in the form of protein was not intended until it was established that with the former type of ration some degree of success could be obtained. However, there is ample evidence in the literature (Slonaker, '38; McCoy, '47; Goettsch, '49) that rations containing 14 to 16% of protein support the attainment of much greater weight of the young at three weeks of age than was observed in these studies even though the concentration of the "essential amino acids" is much lower. Among conditions which may be responsible for the impaired reproductive and lactation performance could be an "imbalance" of the amino acids (Ramasarma et al., '49; Flodin, '53), inadequate utilization of the D-amino acids for biosynthesis of the "nonessential amino acids," a dietary requirement for some of the nonessential

amino acids during the stress of pregnancy and lactation, or the existence of unrecognized nutrients which may be necessary under these conditions. These possibilities can now be investigated by modifications of those diets with which the best results were obtained in this study.

Reproductive performance of the F_2 generation. Three F_2 generation rats were fed ration AA_3 plus 3 gm of DL-isoleucine (Mann, lot 103331) per kilogram of ration since weaning. They cast three litters with a total of 12 young, all of which were weaned at 28 days, with a mean weight of 22.0 gm. Of 4 F_2 generation females fed ration AA_4, one died at term with 8 fetuses in utero; the other three cast a total of 4 litters with 18 young, all of which were weaned at 28 days of age, with a mean weight of 28.0 gm. While the numbers of animals involved are small, the fact that none of the F_3 generation young died does not support the view that rats require a dietary source of an "animal protein factor" for survival of the young.

Miscellaneous observations. With a few rats born to mothers fed rations AA_3 or AA_4 exploratory tests were made to determine if various additions to ration AA_3 would improve reproduction and lactation. Although the numbers of animals involved in the first three groups are small, the results shown in table 6 give some indication concerning the effectiveness of the supplements used. When 0.3% of DL-isoleucine was added to ration AA_3 all of the 25 young in 5 litters survived (group 1). Their average weaning weight, however, was still subnormal and the mothers lost weight during lactation. The addition of liver concentrate alone (group 2) or with DL-isoleucine (group 3) increased the weaning weight of the young although it was still subnormal. The small number of young present in each litter of groups 2 and 3 can account for their greater preweaning weight gains. When purified soybean protein replaced half of the nitrogen contributed by amino acid mixture I to ration AA_4 the resulting ration was improved with respect to the number of young born per litter, the weaning weight of the young and the

maintenance of weight of the mother during lactation (group 4). This indicates that with respect to reproduction the soybean protein or its component amino acids improved the ration to an extent not accomplished by an equal quantity of the "essential amino acids."[6]

TABLE 6

Effect of supplements[1] *on reproductive performance of rats*[2] *fed rations* AA_3

GROUP	SUPPLEMENT PER KG RATION	NO. OF FEMALES	NUMBER OF YOUNG		MEAN WEIGHT OF YOUNG AT 28 DAYS	MEAN WT. CHANGE OF MOTHER IN 28 DAYS POST PARTUM
			Born	Weaned at 28 days		
					gm	*gm*
1	3 gm DL-isoleucine	5	25 (5)[3]	25 (5)	22.0	— 6.0
2	0.4% liver concentrate	4	11 (3)	5 (2)	31.5	— 6.5
3	0.4% liver conc. + 3 gm DL-isoleucine	2	5 (2)	5 (2)	34.0	— 2.0
4	64 gm purified soybean protein	7[4]	69 (10)	58 (9)	37.8	+ 8.0

[1] See table 4, group 3, for reproductive performance of unsupplemented control group.

[2] All rats were young from mothers fed ration AA_3 or AA_4.

[3] Number of litters born in parentheses.

[4] Three of these rats, while fed ration AA_4, had previously cast 4 litters with 23 young of which 13 survived to 28 days of age.

Several mothers were autopsied after completion of lactation. In most instances, the livers were greatly enlarged, pale colored, mottled and friable, suggesting severe fatty infiltration. This condition was not observed in animals of similar age that had failed to conceive or in those that died at parturition. It may be related to the production of fatty livers observed by Harper et al. ('54) as a result of dietary im-

[6] The term "essential amino acid" as defined by Rose ('37) refers only to the dietary requirements of the rat for growth.

balance of amino acids. Analyses of the livers will be reported later.

The long bones of three rats fed ration AA_4 were analysed for ash and calcium. The bones were removed 28 days postpartum after these rats had nursed litters with a total of 19 young. The ash averaged 66.7% and the calcium 25.5% of the fat-free, dry bones. The corresponding values for the bones that were removed from 5 rats fed the rolled oats-casein ration after they had completed a period of intensive lactation were 65.7 and 25.2% respectively. It is evident that the introduction into the ration of some of the amino acids as hydrochlorides and the stress of lactation did not demineralize the skeleton and thus contribute to inadequate lactation.

SUMMARY

1. Protein-free rations containing 8 or 16% of a mixture of 10 amino acids and as much as 97% of compounds of known chemical structure were fed to rats for two, and in a few cases for three generations.

2. With rations containing 8% of the 10 "essential amino acids" and 5.1% of ammonium citrate, there was poor growth, a relatively high incidence of maternal deaths, a subnormal number of young per litter, a high mortality and subnormal growth rate of the young. Correction of a partial isoleucine deficiency increased the survival of the young.

3. With a ration containing 16% of the 10 essential amino acids and 5.1% of ammonium citrate, there was a low incidence of maternal deaths, an increased survival of the young and an increased, though still subnormal, growth rate of the young.

4. The substitution of purified soybean protein for half of the amino acids in the 16% amino acid ration improved lactation.

5. When a ration containing 12.2% of a mixture of 16 amino acids was fed to young rats from weaning, 96% of the young survived the preweaning period but their growth rate during that time was subnormal.

6. The quantitative requirements of the rat for amino acids during reproduction and lactation remain to be defined, but these experiments as yet provide no support for the alleged occurrence of an unknown "animal protein factor" needed for survival of the young.

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ANTAGONISM OF FRESH FAT TO THE TOXICITY OF HEATED AND AERATED COTTONSEED OIL [1]

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TWO FIGURES

It has previously been shown (Kaunitz and Slanetz, '50; Kaunitz, '53) that the inclusion in a complete rat diet of lard heated and aerated for 300 hours at 90°C. produces only mild toxicity symptoms. In the current paper, it will be shown that similarly treated cottonseed oil is severely toxic and that this effect can be counteracted by fresh oil.

PROCEDURE

The studies were carried out on albino rats from a homogeneous colony and were begun when the rats were 4 weeks old. The maintenance diet of the colony and the procedures used to obtain comparable groups have been previously described (Kaunitz et al., '54).

Samples of a commercially available refined cottonseed oil [2] were aerated and heated in a water bath at 90 to 95°C. for 50 to 300 hours. A clear oil always resulted. The peroxide numbers were repeatedly determined by the Stansby procedure.

The desired amounts of the treated or fresh oil or both, alcohol-washed casein and cerelose comprised 93.5% of the

[1] Aided by a grant from the Schenley Laboratories Inc., New York, N. Y.

[2] Wesson Oil.

purified diet employed. To these constituents were added 4% salt mixture (U.S.P. no. 2), 0.5% calcium carbonate, and 2% calcium carbonate, and 2% celluration and, per kilogram, 1 gm of inositol, 1 gm of choline, 300 mg of para-aminobenzoic acid, 100 mg of nicotinic acid, and 10 mg of vitamin K. Other food factors were supplied by feeding 4 times weekly two drops of a watery suspension containing, per milliliter, 4 mg of thiamine, 8 mg of riboflavin, 8 mg of pyridoxine, 20 mg of calcium pantothenate, 5 mg of folic acid, 0.05 mg of biotin, 10 μg of vitamin B_{12}, and 50 mg of ascorbic acid. The fat-soluble factors were administered in a linoleic acid suspension containing, per milliliter, 50 mg of alpha-tocopherol acetate, 10 mg of free alpha-tocopherol, 0.5 mg of vitamin D_2, and 5 mg of crystalline beta-carotene.[3]

In the course of the experiments, it became necessary to pair-feed rats on diets containing the treated oil with matching groups receiving fresh oil in addition. The compositions of the rations containing fresh oil were adjusted so that isocaloric amounts of the diets to be compared would contain identical amounts of protein and of treated oil. For instance, when 15% treated oil and 30% protein were used, 1 gm of the diet was equivalent to 4.49 calories. A diet containing 30% fat was equivalent to 5.27 calories per gram. Therefore, an amount of the high-fat diet equal to 85.2% of a given amount of the low-fat diet had to be fed. In order to make the protein and treated oil intakes of the animals on the two diets equal, $\frac{100}{85.2} \times 30\%$ protein and $\frac{100}{85.2} \times 15\%$ treated oil were used in the high-fat diet which thus contained 35.2% protein, 17.6% treated and 12.4% fresh oils. This procedure is acceptable only if it can be assumed that there was no significant difference in the calories lost in the feces of the paired groups. Despite the diarrhea observed in animals eating the treated

[3] Dr. Leo Pirk of Hoffmann-La Roche, Inc., Nutley, New Jersey, generously supplied us with most of the synthetic vitamins used. Vitamin D_2 was supplied by the Sterling-Winthrop Research Institute, Rensselaer, N. Y., and the crystalline beta-carotene, by the Barnett Laboratories, Long Beach, California.

oil, it is highly improbable that caloric losses need consideration in the evaluation of the experimental results.

The rats were kept in single unit cages with wire bottoms and removable pans to facilitate the determination of food consumption. Body weights were recorded by plotting the logarithm of the weight against the reciprocal value of the age (Zucker and Zucker, '42).

The organ weight — body weight relationship has been presented as a log — log plot. To obtain data on normal rats, the organs of 130 male rats varying in body weight from 18 to 450 gm were examined. These animals had been on a complete diet containing 10% lard and 30% casein and were considered normal because animals on this diet grew normally (according to Zucker and Zucker, '42) and males remained fertile during this period. On the log — log plot, the upper and lower limits of the spread formed parallel lines. For brevity and clarity, only these lines and not the individual points for the normal rats are given below for the comparison with the experimental animals.

RESULTS

When rats were fed a diet containing 30% casein and 15% of cottonseed oil which had been aerated and heated to 95°C. for 200 hours, their daily caloric intake was less than half of that of rats receiving fresh fat or no fat at all. They began losing weight at once (fig. 1a, curve III). Three weeks after having been placed on the diets containing 15% of the treated material, 12 to 75% (on the average, roughly half) of 4 groups had died (table 1). When only 10% of the treated oil was used, few rats had died among 6 groups three weeks after the experiment started. They were somehow able partly to adjust to the treated oil in that they gradually lost their diarrhea (see below) and even increased in weight slightly (fig. 1b). The circumstance that the weight increase though present, was very small even in the absence of the diarrhea suggests that the weight reduction was not due primarily to diarrhea.

With 20% of the treated material, all rats died within three weeks. Similar results have been obtained with linseed oil heated to 275°C. in the absence of oxygen (Crampton et al., '51). The rats had the appearance of starving animals with

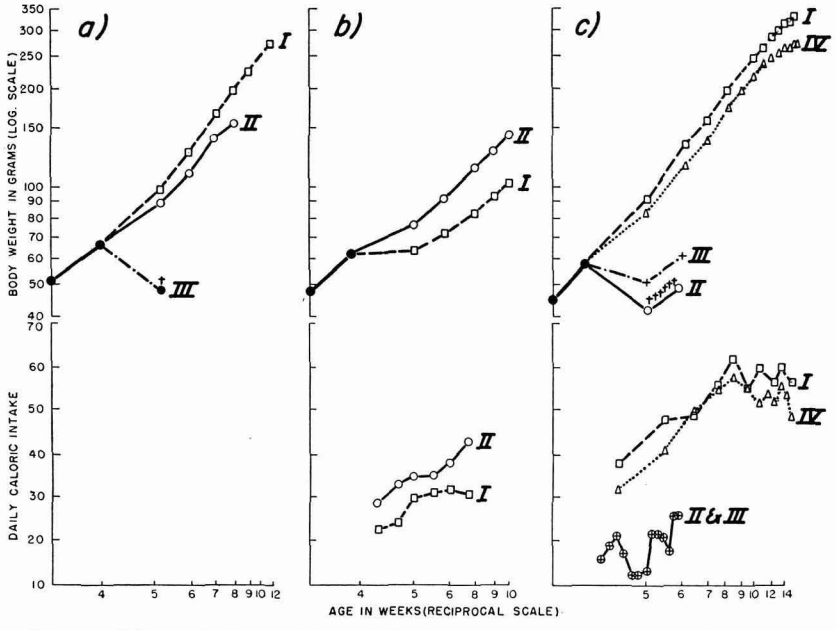

Fig. 1 Effect of the addition of fresh or "treated" cottonseed oil or both on growth and food consumption of albino rats fed diets containing 30 or 60% of casein. The "treated" cottonseed oil was aerated and heated to 95°C. for 50 to 300 hours.

(a) averages of 8 males fed 30% casein and
- I 20% fresh oil
- II 20% mildly treated oil (peroxide number: 191)
- III 20% more strongly treated oil (peroxide number: 141)

(b) averages of 12 females fed 60% casein and
- I 10% treated oil
- II 10% treated and 20% fresh oils

(c) averages of 16 males fed 30% casein and
- I 10% oil
- II 15% treated cottonseed oil
- III 17.6% treated and 12.4% fresh oil with 35.2% casein (pair-fed with II)
- IV fat-free with 2% linoleic acid

dirty, and sometimes sparse, fur. They had severe diarrhea, the feces containing appreciable amounts of mucus and being lighter in color than those of the controls. Histological examinations of nearly all organs [4] of 6 rats in an advanced stage of the disease failed to reveal any abnormalities ex-

TABLE 1

Effect of the addition of fresh or "treated" cottonseed oil or both on growth and food consumption of albino rats fed diets containing different amounts of casein. The "treated" oil was aerated and heated to 95°C. for 50 to 300 hours.

NO. AND SEX	TREATED OIL ONLY			TREATED OR FRESH OIL OR BOTH			
	% treated oil in diet	% casein in diet	% dead after 3 weeks on diet	% treated oil in diet	% fresh oil in diet	% casein in diet	% dead after 3 weeks on diet
7 ♂ [1]	10	5	29	...	10	5	0
7 ♂ [1]	10	30	0	...	10	30	0
16 ♀	10	30	0	10	15	30	0
8 ♂ [1]	10	49.5	12	12.8	17.2	63.5	0
12 ♀	10	60	0	10	20	60	0
7 ♂	10	74	43	...	10	74	0
16 ♂ [1]	15	30	38	17.6	12.4	35.2	0
				17.6	12.4	35.2	0
16 ♂ [1]	15	30	63	15.9	4.1	31.7	0
				17.6	12.4	35.2	0
8 ♂ [1]	15	54	12	15	15	63.5	0
8 ♂ [1]	15	54	75	17.6	12.4	63.5	25
8 ♂	20	5	100				
8 ♂	20	30	100				
8 ♂	20	30	100				

[1] Pair-fed with animals on the same line.

cept mild edema of the intestinal mucous membrane in some instances.

When fresh oil was added to the diet containing the treated oil, the effects of the latter could be nullified to a large extent. Male rats receiving 15% of the treated and 15% of the fresh oils and permitted to eat without restriction were alive three months after the experiment had started and showed no

[4] We are greatly indebted to Dr. Herbert Stoerk of the Merck Institute for Therapeutic Research, Rahway, New Jersey, for the histological studies reported in this paper.

signs of the disease except that their body weights were well below those of the rats receiving only fresh fat. This result was similar to those obtained with heated and aerated lard in which decreased growth was the only sign of abnormality. When female rats receiving 10% of the treated cottonseed oil were mated after several weeks on the diet and after they had been without diarrhea for several weeks, they either did not become pregnant or resorbed the fetuses. With fresh cottonseed oil in addition to the treated material, nearly normal litters were born and reared. The beneficial effects of adding fresh oil to diets containing treated material were the more remarkable because the higher food intake of the animals receiving supplements of fresh fat would result at the same time in an increase in the intake of heated and aerated oil (fig. 1 b). Paired feeding experiments demonstrated that the very low caloric intake of the freely-eating group fed treated oil was not responsible for most of the toxic symptoms observed. The animals receiving isocaloric amounts of the diet containing fresh as well as treated oil grew better and lived longer than the animals with which they were paired (fig. 1 c); this was ascertained in three separate experiments.

Determination of the hemoglobin content and counts of the red and white blood cells were made on pair-fed animals receiving 5 to 74% protein and 15% treated oil with and without fresh oil. There were no essential differences among the groups as to hemoglobin and red blood cells. Among 15 animals receiving 30% casein and 15% treated oil, 7 had white counts of over 9000 per cubic millimeter, while none of their pair-fed controls receiving fresh oil had counts this high. Despite great individual variations, there was a similar trend among the animals receiving 5 and 74% casein.

Organ weight studies are shown in figure 2. The curve of the normal kidney (fig. 2 a) revealed a break at 40 to 50 gm body weight, which corroborates the findings of previous workers (Stoerk and Zucker, '46). It is evident that nearly all kidneys from animals on treated oil are considerably outside the upper limit for normal kidneys while the weights

of those receiving fresh and treated oil are within normal limits. In fact, the kidney weights of the animals fed only the treated oil were heavier than those of normal rats weighing 60 gm, which was the average weight of the groups just be-

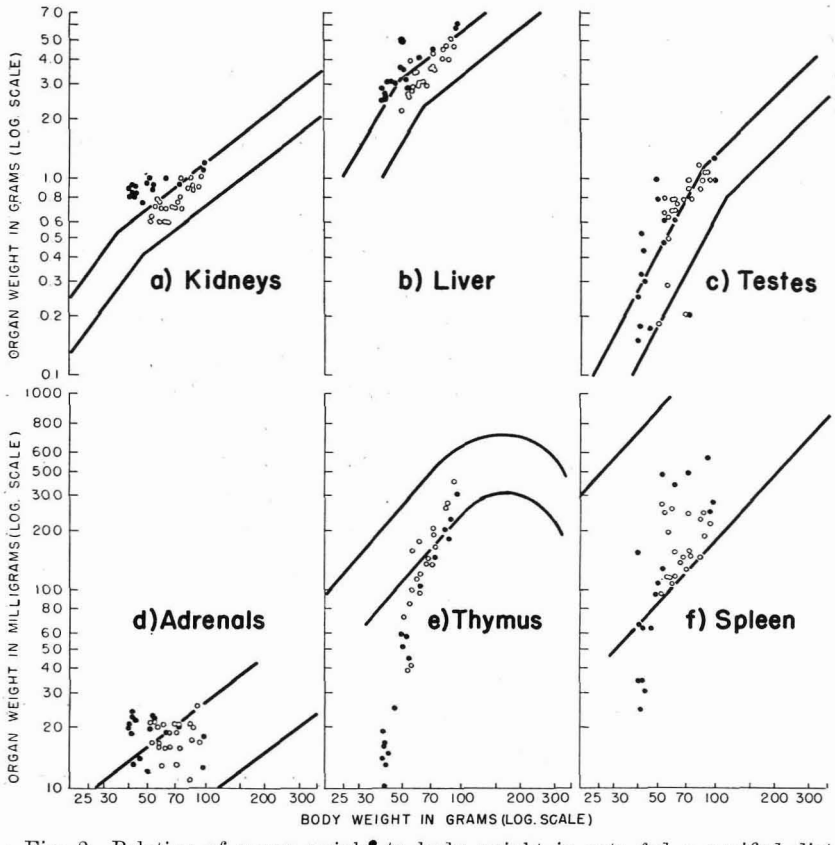

Fig. 2 Relation of organ weight to body weight in rats fed a purified diet containing 60% casein and refined cottonseed oil aerated and heated to 95°C. for 200 to 300 hours. The closed circles refer to animals freely eating a diet containing 15% of the treated oil; the open circles, to animals pair-fed with the latter and receiving 17.6% treated and 12.4% fresh oils. The parallel lines indicate the limits of variation in the organs from 130 normal animals.

fore the administration of the treated oil. Thus, while the body weight declined, the kidneys increased in size. Simultaneous administration of fresh oil kept the relation of kidney to body weight within normal limits.

The liver weights (fig. 2 b) showed similar, but somewhat less pronounced, changes. Most of the livers of animals on treated oil were well above those of normal rats; fresh oil prevented the changes. Organ and body weights of rats fed rancid lard have been reported by Greenberg and Frazer ('53). Their average results for kidneys and livers of animals fed rancid or fresh lard fall within the range for normal rats as given in figure 2a and b. It is true, however, that the liver and kidney weights for the rats fed rancid lard tend to be on the upper limit for normal rats while those of the animals fed fresh lard are lower. This is further evidence that heated and aerated lard is much less toxic than similarly treated cottonseed oil.

The scattering of the normal adrenal weights was much greater than that of the liver and kidneys (fig. 2 c). However, in 9 out of 16 cases, the adrenals of those fed treated oil exceeded the maximum weight of the normals while, in the group fed fresh as well as treated oil, this occurred only 4 times.

The majority of the testicular weights of both groups of experimental animals were above those of the normal ones (fig. 2 d). In many instances, therefore, the testes did not lose weight, and some even increased in size despite the loss in body weight resulting from the feeding of the treated oil. This was not changed by the addition of fresh oil to the diet.

The shape of the thymus — body weight relationship in normal animals (fig. 2 e) expresses the involution of the thymus. The curve found in these studies is not appreciably different from that described by Stoerk ('46). Extremely small thymuses were found in the majority of animals fed treated oil. The thymuses of about half of the pair-fed controls receiving treated and fresh oils were also smaller than normal (presumably as an expression of semi-starvation) but were much larger than those fed only the treated oil.

The variation in splenic weights among the animals far exceeded the variations in any of the other organs. Yet, it seems evident that the spleens of about half of the animals fed only treated oil are below the lower limit for normal

spleens while those of their pair-fed controls are just within normal limits.

In further studies, an attempt was made to modify the toxic effect of the treated oil by feeding extra amounts of the fat-soluble vitamins and by the feeding of fresh oil by dropper rather than by inclusion in the diet.

When animals receiving 15% of the treated oil were given daily feedings by dropper of about 25 mg of synthetic DL-alpha-tocopherol acetate containing 125 μg of crystalline beta-carotene and 2.5 μg of vitamin D_2, their average weight after two weeks was 75 gm, while that of the rats receiving no supplement was 61, the difference being statistically significant. However, a group receiving 15% of the treated oil and 10% of fresh oil weighed 99 gm on the average at this time. Therefore, the mildy protective effect of tocopherol (and the other fat-soluble factors) does not explain the strongly protective action of fresh oil added to the diet.

Daily feeding by dropper of 0.5 to 0.8 gm of fresh, refined cottonseed oil exerted only a mild protective effect as to growth and survival time in rats fed 15% of the treated oil. After three weeks on the experimental diet, those receiving no supplement weighed, on the average, 60 gm and three out of 12 had died; those fed fresh oil in addition weighed, on the average, 69 gm and none had died. However, when a comparable amount of fresh oil was added to the diet, the average weight was well above 100 gm. We have so far no explanation for this difference.

DISCUSSION

In studies on heated and aerated lard (Kaunitz and Slanetz, '50), it was concluded that the amount of peroxides present is not related to the degree of toxicity. In the current studies, similar results were obtained. In the experiments shown in figure 1a, it was found that a sample of cottonseed oil, heated and aerated to a peroxide number of 191, brought about only very mild growth retardation. When heating and aeration were continued, the peroxide number dropped to 141, but the

sample had become very toxic. It would therefore seem that, if the toxic products are the result of oxidation, they may be breakdown products of peroxides; but it is quite possible that the toxic products are not at all, or only partly, related to oxidative changes. Crampton and his co-workers ('53), in their studies on heated linseed oil advanced this latter viewpoint. They made it seem probable that some products formed during thermal polymerization are toxic.

Concerning the antagonism between treated and fresh oils, we had first believed that an antimetabolite relationship might exist between the toxic products and fresh oil. However, in view of the fact that the protective effect of fresh oil is only mild when it is not added to the diet but is fed separately, the antimetabolite theory may not be a good explanation for the facts observed. The possibility must be considered that addition of fresh oil to heated oil may change the toxic properties of the latter, perhaps by influencing the state of polymerization.[5] But whatever explanation may eventually prove to be correct, it seems noteworthy that fresh oil may, under certain conditions, exert a life-saving effect which is not due to its caloric properties.

SUMMARY

1. The inclusion in a rat diet of 15 to 20% of refined cottonseed oil, aerated and heated to 95°C. for 200 to 300 hours, led to rapid loss of weight and death within three weeks.

2. The condition was accompanied by diarrhea and by the occurrence of large livers, kidneys, and adrenals and small spleens and thymuses. Histologically, the only change was an occasional intestinal edema.

3. Addition of fresh oil to the diet containing the heated and aerated oil protected the animals against the toxicity. Only growth retardation persisted. This protective effect

[5] This possibility was discussed with a number of workers in the fields of fat and of polymer chemistry. While they agreed that no definite opinion could be given, some of the workers felt that at least changes in the kind of polymerizations could be effected by the addition of fresh to polymerized oil.

could also be observed in paired-feeding experments in which the paired rats received the same number of calories and equal amounts of protein and of treated oil. When fresh cottonseed oil was fed by dropper instead of being included in the diet, its protective effect was only slight.

4. Extra feeding of 25 mg of DL-alpha-tocopherol acetate (and other fat-soluble factors) gave mild protection.

5. Peroxides are probably not responsible for the toxic effect of the heated and aerated cottonseed oil; polymerization may be a better explanation. No definite explanation for the antagonism of fresh to treated oil can be given; the effect is probably not due to an antimetabolite relationship but could be caused by a change in the state of polymerization.

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OBSERVATIONS ON THE PRODUCTION OF SMOOTH-SURFACE RAT CARIES BY DIETS CONTAINING SKIMMILK AND WHEY POWDERS

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The development of smooth-surface caries in white rats as a result of feeding a complex diet containing heat-processed cereal foods was previously reported (McClure, '52). Also, in a comparison of diets containing roller-process skimmilk powders with a diet containing a spray-process powder, the highest incidence of caries and the most severe caries resulted from diets containing the roller-process powders (McClure and Folk, '53). In evaluating this later result, account was taken of the fact that a more severe heating is generally applied during roller drying than during the spray drying of liquid milk (Hunzicker, '49; Whittier and Webb, '50). Furthermore, it was shown that an additional heat treatment, brought about by the "dry autoclaving" of these two commercial powders, increased this development of smooth-surface caries (McClure and Folk, '53). The over-all results thus raised the question as to what extent the effect of heat processing on skimmilk powders was a factor in this production of experimental rat caries. In subsequent studies a diet containing a spray-process skimmilk powder stored at 37°C. and 70% relative humidity for 90 days was significantly more cario-

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genic than a diet containing the parent powder (Folk and McClure, '54). This result suggested that storage conditions also may bring about changes in a milk powder which may contribute to a cariogenic effect in a rat diet.

It is the purpose of this report, therefore, to present additional data bearing on the possibility of a relation of heat processing of skimmilk powders to the development of smooth-surface caries in white rats and also to present preliminary results showing the production of smooth-surface caries by diets similar to those containing milk powders, excepting that the milk powder was replaced by a protein-equivalent quantity of purified casein and a commercial whey powder.

EXPERIMENTAL

In the study of milk powders the products used were as follows: powder "X" (diet 666) was prepared by a freeze-dry process [2] and at no time did the temperature of the original milk or powder exceed 40°C. This powder was white and very flaky. Skimmilk powder "A" (diet 633) was an "improved low-heat spray process non-fat powder"; skimmilk powder "C" (diet 635) was a roller-process non-fat powder. In the study of whey powders, diet 661 contained a commercial roller-process powder ("A") made from cheddar cheese whey. This product comes off the rollers in large chunks and was *finely ground before feeding*. Whey powder "B" (diet 662) was prepared by autoclaving whey powder "A" for 15 minutes at 15 pounds pressure in an open pan. It was dried overnight at 90°C. and ground fine. Whey powder "C" (diet 667) was a spray process "edible dry cheese whey." As obtained commercially it was a fine powder. Whey powder "D" (diet 668) was prepared by autoclaving powder "C" for 15 minutes at 15 pounds pressure in an open pan. It was dried overnight at 90°C. and ground to a fine powder. It should be noted particularly regarding the commercial preparation of whey powders, that a number of variable procedures, in addition to the method of drying, may influence the characteris-

[2] Prepared by Ben Venue Laboratories, Inc., Bedford, Ohio.

tics and quality of the product ((Hunzicker, '49; Whittier and Webb, '50). All the milk powders and the diets were stored in closed containers at 5°C. prior to use.

The whey powders, present at a level of 25% in diets 661, 662, 667 and 668, supplied 3 to 4% of the dietary protein in the form of whey protein. The total protein content was adjusted to the 13.5% level of the skimmilk-powder diets (666, 663 and 635) by addition of 10% of vitamin-free casein. The whey-powder diets contained therefore, essentially 35% of a reconstituted skimmilk powder.

The components of the complete diets and analytical data relative to the diets are shown in table 1. In addition to the diet, the rats were given weekly by mouth a vitamin supplement which provided approximately 1500 units of A, 100 units D_2, and 5 mg of alpha tocopherol. They drank distilled water and ate ad libitum. Fresh supplies of diets 666, 663 and 635 were fed each day and all refused portions were discarded. In the studies with the whey-powder diets, food was renewed in the food cups at 3- to 4-day intervals and refused food was not removed.

The rats were at weanling age when started and were housed two per cage on screen bottoms. Holtzman and Sprague-Dawley strains were represented equally in all the groups. Litter mate trios were fed diets 633, 635 and 666, litter-mate pairs, diets 661 and 662 as well as diets 667 and 668. Males and females were equally distributed in all experiments. No strain or sex differences have been observed in any of the results. At the termination of the experiments the rats were killed, the heads autoclaved, soft tissues removed, and caries was diagnosed as previously described (McClure and Folk, '53).

RESULTS

Pertinent data on management and growth of the rats and results of the caries diagnosis pertaining to the skimmilk powder diets are shown in table 2. Diet 666, containing a lyophilized skimmilk powder produced a low incidence (15.4%)

TABLE 1

Composition and analysis of diets

DIET NO.	666	633	635	661	662	667	668
SKIMMILK OR WHEY POWDER	X	A	C	A	B	C	D
	%	%	%	%	%	%	%
Skimmilk powder	35.0	35.0	35.0	...	...	...	...
Whey powder	...	...	...	25.0	25.0	25.0	25.0
Cerelose	18.0	18.0	18.0	18.0	18.0	18.0	18.0
Cornstarch	45.0	45.0	45.0	45.0	45.0	45.0	45.0
Casein	...	...	...	10.0	10.0	10.0	10.0
Dry liver powder	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Dry substance	90.15	91.25	90.70	92.50	92.50	91.78	91.70
Protein (N × 6.25)	13.10	13.65	13.50	13.50	13.76	13.35	13.50
Fat	0.32	0.21	0.22	0.20	0.20	0.35	0.31
Ash	2.79	2.93	2.93	2.47	2.39	2.25	2.22
Calcium	0.48	0.54	0.53	0.27	0.21	0.18	0.17
Phosphorus	0.36	0.39	0.38	0.30	0.28	0.28	0.28
Fluorine (p.p.m.)	(0.25)	(0.20)	(0.30)	(0.41)	(0.64)	(0.52)	(0.51)
Lactose [1]	18.2	18.1	17.6	16.0	14.8	17.6	15.3

[1] Soluble lactose in the powders was determined as total anhydrous lactose by a polarographic method. These lactose results are as follows: milk powder "X" 51.9%, "A" 51.8%, "C" 50.2%; whey powders "A" 64.0%, "B" 59.1%, "C" 70.5%. "D" 61.5%.

of caries with an average score of 0.5. On diet 633, containing a commercial spray-process skimmilk powder, incidence of caries, average number of carious teeth and the average caries score were about three times those on diet 666. Of

TABLE 2

Caries experience in rats fed diets containing different dry skimmilk powders

DIET NO.	666	633	635
MILK POWDER PREPARATION	LYPHOLIZED	SPRAY	ROLLER
No. of litters	36	36	36
No. of rats	39	38	39
Days on experiment	91	91	91
Initial wt. (gm)	26.2	26.2	26.9
Final wt. (gm)	177.8	203.4	173.2
Av. daily gain (gm)	1.7	2.0	1.6
Caries incidence — Per cent			
Carious rats	15.4	42.1	66.7
Occlusal carious rats	0.0	0.0	10.3
Surface carious rats	15.4	42.1	66.7
Caries distribution and severity — no. per rat			
Upper carious teeth	0.0	0.0	0.0
Lower carious teeth	0.3	0.8	2.0
Total carious teeth	0.3	0.8	2.0
Occlusal carious teeth	0.0	0.0	0.2
Surface carious teeth	0.3	0.8	1.8
Occlusal carious areas	0.0	0.0	0.3
Lingual carious areas	0.0	0.0	0.0
Buccal carious areas	0.5	1.4	3.1
Score per rat	0.5	1.4	3.5
Score per carious rat [1]	3.2	3.3	5.1

[1] Total group score divided by number of carious rats in the group.

the skimmilk powder diets the highest incidence and most severe caries resulted from diet 635 which contained a roller-process skimmilk powder.

The data pertaining to the whey powder studies are shown in table 3. On diets 667 and 661, containing commercial whey powders, approximately 50% of the rats developed caries.

Although comparison of these two whey powder diets is not entirely justified owing to the fact that rats fed the two diets were not litter mates, severity of caries was greater on diet 667 than on 661. Diets 662 and 668 contained autoclaved

TABLE 3

Caries experience in rats fed diets containing different dry whey powders

DIET NO.	667	668	661	662
WHEY POWDER PREPARATION	SPRAY	SPRAY [1]	ROLLER	ROLLER [1]
No. of litters	21	22	20	20
No. of rats	38	38	38	37
Days on experiment	88	88	90	90
Initial wt. (gm)	26.2	26.1	29.4	30.2
Final wt. (gm)	179.8	123.6	205.4	136.7
Av. daily gain (gm)	1.8	1.1	2.0	1.2
Caries incidence — Per cent				
Carious rats	52.6	63.2	47.4	78.4
Occlusal carious rats	0.0	7.9	0.0	0.0
Surface carious rats	52.6	63.2	47.4	78.4
Caries distribution and severity — no. per rat				
Upper carious teeth	0.1	0.1	0.0	0.0
Lower carious teeth	1.7	1.9	1.1	2.0
Total carious teeth	1.8	2.0	1.1	2.0
Occlusal carious teeth	0.0	0.2	0.0	0.0
Surface carious teeth	1.8	1.9	1.1	2.0
Occlusal carious areas	0.0	0.3	0.0	0.0
Lingual carious areas	0.0	0.2	0.0	0.0
Buccal carious areas	3.2	2.8	1.8	3.6
Score per rat	4.2	3.8	1.8	4.5
Score per carious rat [2]	7.9	6.1	3.7	5.7

[1] Autoclaved powder.

[2] Total group score divided by number of carious rats in the group.

whey powders, one of which apparently had its cariogenic properties aggravated by autoclaving (i.e., powder "B" in diet 662), whereas the other diet, containing autoclaved whey powder "D" (diet 668), did not differ, as regards its cariogenicity, from diet 667 containing the parent powder. Compar-

ing diets 667 and 668, chi-square for the difference in caries incidence was 0.70 and Fisher's t-value for paired differences in caries scores was 0.57.

As shown by data in tables 2 and 3 very few rats developed carious lesions in upper teeth and most of the lesions occurred on buccal surfaces. The appearance of this type of smooth-surface caries has been described in previous reports (McClure, '52; McClure and Folk, '53). Occlusal-fissure caries was present to a very limited extent and only in rats fed diets 635 and 668.

In the absence of controlled food intakes, small differences in the data for average daily gains are not significant. A sizeable reduction in growth, however, seems apparent in the weight data for rats given diets 662 and 668 which contained autoclaved whey powders. There is no certain explanation as to the cause of these growth failures.

DISCUSSION

The data presented in table 1 show that quantities of basic constituents of these diets were generally quite uniform. Variations in total protein, fat, ash, calcium, phosphorus and fluorine were not related to caries results. All of the diets contained approximately 33 to 35% of total sugar in the form of cerelose and lactose. Analyses for total soluble lactose (Sharp and Doob, '41) in the skimmilk and whey powders, indicate losses of lactose in whey powders due to autoclaving. Total quantities of lactose in the diets, however, do not correlate with the caries data.

Milk powder "X", used in diet 666, was not heated during the course of its preparation and with the exception of 3 to 4 days during shipment to our laboratory, was stored under refrigeration prior to its incorporation into diet 666. In addition diets 666, 633, and 635 were kept refrigerated and fresh diet was supplied to the rats daily to avoid deterioration during the course of the experiment (McClure and Folk, '54). Nevertheless, 6 out of 39 rats on diet 666 developed smooth-surface caries. Thus it would appear that although pre-

cautions were taken to avoid deterioration of the milk powders, this diet as fed possessed an inherent degree of cariogenicity. Since the milk powders used in diets 666, 633 and 635 were all from different sources the possibility exists also that factors other than heating could contribute to the increase in caries observed with the two latter diets. In any event the results agree with previous observations (McClure and Folk, '53) that diets containing the roller-process skimmilk powders were more cariogenic than a diet containing a spray-process powder. Thus, while commercial heat treatment of the skimmilk powders may not be the sole condition responsible for the basic cariogenicity of these skimmilk powder diets, it does appear to have a significant effect in increasing their cariogenicity.

On diets in which the skimmilk fractions have been replaced by protein-equivalent amounts of a spray-dried or roller-dried whey powder and unheated vitamin-free casein, the rats exhibit the same general pattern of smooth-surface caries as those fed the heat-processed skimmilk powder diets. If it can be assumed that it is the heat processed component of these diets which is largely responsible for the observed caries results, the fact that this cariogenic effect can apparently be associated with the whey fraction becomes of considerable importance.

The whey powders contributed 3 to 4% of the protein and all of the lactose to diets 661, 662, 667 and 668. In previous experiments, in which casein constituted the sole source of protein in the diet, smooth-surface caries did not develop (McClure, '45), although these diets also did not contain lactose. The information presently available thus does not permit further identification of the factor or factors responsible for the cariogenicity of these diets.

As in the case of milk powders, heating *per se* may not be the only factor contributing to the cariogenicity of the whey powder diets, judging from the results with diet 668 in which autoclaving of the spray-dried whey powder did not result in an increase of caries over that found on diet 667

which contained the same whey powder unautoclaved. In contrast, rats fed diet 662 containing an autoclaved roller-process whey powder showed a far greater incidence and a much increased severity of smooth-surface caries than those on diet 661 which contained the same whey powder unautoclaved. Explanation of these findings is complicated by the fact that the two whey powders were obtained from different sources as well as by the previously mentioned possibility of wide variations in quality and processing procedures. It may be noted that autoclaving as an additional heat treatment likewise may be questioned as a procedure which does not duplicate the changes which usually result from commercial heat processing. In this connection a statement by DeBaun and Connors ('54) is pertinent, i.e., that "the high heat of roller drying may induce a different mechanism of protein sugar reaction, than prolonged storage at lower temperatures." It is obvious that further study will be required on these aspects of the problem.

Thus far in our experience smooth-surface caries has consistently appeared in rats fed diets containing processed cereals, skimmilk powders, and in the present experiments with diets in which whey powders were combined with unheated casein. Although an explanation of these results obviously calls attention to the complex chemical changes and nutritional losses in protein which are brought about by heat processing (Griswold, '51; Melnick and Oser, '49; Patton, '50; Fairbanks and Mitchell, '35; and Patton and Flipse, '53), there is no evidence of any correlation of the better known of such changes with this production of experimental rat caries. In the present study there does not appear to be any association of growth results with the dental caries experience. It would appear, however, that these findings offer a promising basis for future experimentation which will be directed toward the study of the interactions of specific milk proteins and lactose on heating, and their possible relationship to this experimental caries production. The fact that this type of smooth-surface experimental caries closely resembles human smooth-

surface caries further justifies continued study of these diets in animal caries experimentation.

CONCLUSIONS

1. Rats fed diets in which the protein was supplied in the form of three different skimmilk powders, developed smooth-surface caries which occurred predominantly on lower buccal areas.

2. The incidence of caries and the average number of teeth affected, varied in a manner which paralleled the severity of the heat treatment necessary for the preparation of the milk powders. Diets containing spray-process and roller-process powders were both much more cariogenic than a diet containing a lypholized milk powder. The most severe cariogenicity was associated with the diet containing a roller-process powder.

3. When the milk powder diets were modified by replacing the milk powders with a protein equivalent quantity of either a spray-process or a roller-process whey powder and vitamin-free casein, the rats developed the same pattern of smooth-surface caries.

4. Although autoclaving the roller-process whey powder appeared to enhance cariogenicity of diets containing it, this did not pertain to the spray-process whey powder.

5. The results obtained in this study support previous evidence that commercial heat-processing of skimmilk powders may be an aggravating factor of the cariogenic effects shown by diets containing skimmilk powders.

6. The similarity of this experimental rat caries to human smooth-surface caries, further justifies interest in the cariogenic potential of commercial heat-processed foods.

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THE RELATION OF AGE TO FAT ABSORPTION IN ADULT WOMEN TOGETHER WITH OBSERVATIONS ON CONCENTRATION OF SERUM CHOLESTEROL[1]

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FOUR FIGURES

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Information about changes occurring in digestive and absorptive processes with progression of age in man is meager. An atrophy of the salivary glands with advancing years that is followed by a decrease in salivary volume has been reported (Meyer et al., '37). It has also been demonstrated that gastric acidity decreases gradually with age (Polland, '33). The concentrations of certain digestive enzymes such as ptyalin (Meyer et al., '37, '40), pepsin and trypsin (Meyer et al., '40), and pancreatic lipase (Necheles et al., '42) are decreased in elderly people. A diminution in the rate of intestinal absorption of galactose (Meyer et al., '43), glucose (Hofstatter et al., '45; Smith and Shock, '49) and fats (Becker et al., '50) likewise has been shown to occur in the aged.

Considerable disagreement exists regarding the mechanism of fat absorption. Some workers believe that fats are com-

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pletely hydrolyzed prior to absorption from the intestines (Verzar and McDougall, '36; Bloor, '22) while others maintain that a major portion of ingested fats is absorbed unhydrolyzed (Mellanby, '27; Frazer, '40, '43a, '43b; Frazer et al., '44).

Early workers have demonstrated that chylomicron particles in the blood are related to the fat absorbed from the intestines (Gage and Fish, '24; Frazer and Stewart, '37, '39). Gofman and co-workers ('51) believe that these particles are the Sf 40,000 fraction of the blood lipoproteins. Chemical analyses reveal that this fraction consists chiefly of neutral fat, about 7% protein, small amounts of phospholipid, and contains less than 1% of the total serum cholesterol.

At the present time, there is increasing interest in lipid metabolism and its relation to the etiology of atherosclerosis. There is evidence that there may be a link between dietary fat or dietary cholesterol or both and the occurrence of this degenerative disease in man. Such a possibility is an important consideration in the field of nutrition.

The present study was undertaken to investigate the relation of age to the pattern of fat absorption in adult women. Serum cholesterol was also measured. Since both serum cholesterol and the number of chylomicron particles in the blood have been associated with the development of human atherosclerosis, it seemed of interest to find out whether they were related to one another.

EXPERIMENTAL PROCEDURE

The experimental subjects in the present study were 58 apparently normal women ranging in age from 30 to 90 years. There were at least 10 subjects in each decade with the exception of the 9th in which there were only three women.

The standard test meal, given as breakfast during the course of the experiment, consisted of 0.5 gm oleomargarine per kilogram of body weight served on two pieces of toast with a cup of black coffee and a small glass of fruit juice. A blood sample was taken from the fingertip before the test

meal and at hourly intervals thereafter for about 7 hours. A low-fat lunch consisting of a cup of boullion soup, two to three soda crackers, a portion of green beans, peas or celery and carrot strips and a small dish of fruit was served during the course of the day.

Fat absorption was studied by determinations of the concentration of chylomicrons in the blood at successive intervals of time. In the present experiment, slight modifications were made of the techniques reported in the literature (Frazer and Stewart, '39; Becker et al., '50; Frailing and Owen, '51).

A small free-flowing drop of blood obtained from a finger-prick was placed on a coverslip (No. 2), which was then inverted on a clean slide (1.45 to 1.6 mm thick) free from scratches. A thin smear was obtained by pressing down lightly on the coverslip. Excess blood around the edges was wiped off with a piece of lens paper. The coverslip was sealed to the slide with lubricating jelly. Two to 5 such preparations were made and the chylomicrons were counted immediately, using only those preparations which had a uniform distribution of chylomicrons and other blood elements within several microscopic fields.

The dark-field apparatus consisted of a Bausch and Lomb binocular microscope, a paraboloid condenser with an attached dark-field lamp, a 10 × eyepiece and a 97 × (1.8 mm) oil immersion objective. A cardboard-net device inserted into the eyepiece divided the microscopic field into 48 squares. This device facilitated the counting of moving fat particles. Chylomicrons in two to 5 microscopic fields selected from the various preparations were counted and the results averaged per microscopic field. Satisfactory results can be obtained by this method if technical difficulties in counting chylomicrons are recognized and skill developed through practice. It is believed that the present investigator became so skillful that the results obtained are comparable within this study.

Serum cholesterol was determined by a modification of the Sperry and Brand ('43) micro-method for cholesterol. The modification was primarily a reduction in volume of the

sample and reagents by a factor of 10, except for the following changes: 0.1 ml serum sample was diluted to 10 ml and 1 ml aliquots used in the determination. Concentrations of cholesterol in the standards ranged from 0.015 to 0.030 mg per 0.5 ml. Readings were made at 620 mμ with the Beckman spectrophotometer.

RESULTS AND DISCUSSION

The number of chylomicron particles present in the blood stream following the ingestion of fat will depend on the rate of absorption of fat from the intestines and the rate at which fat is being withdrawn from the blood. Factors such as the emptying time of the stomach, the rate of fat hydrolysis, and possibly the rate of chylomicron formation from neutral fats will determine the rate of intestinal fat absorption. The factors involved in the removal of fat from the blood stream are as yet unknown.

Studies in which the concentration of chylomicrons in the blood of human adults was determined indicate that the typical chylomicron curve reaches a peak within two to 4 hours after a fat meal and returns to the initial level at a fairly rapid rate (Gage and Fish, '24; Frazer and Stewart, '37; Becker et al., '50).

In the present study, the chylomicron counts rose to maximum values within one to 6 hours after the test meal whereupon they returned to fasting values. Figure 1 presents the average chylomicron counts at hourly intervals after the test meal for women representing successive decades of life.

Fasting blood samples contained similar numbers of chylomicron particles at all decades of life. In the younger subjects, however, the peak number of chylomicrons was reached one to three hours after the standard test meal and in the older women after 4 to 6 hours. The return to initial values was achieved within 4 to 7 hours in most cases. In a few instances the determinations were not continued long enough to re-attain fasting levels, since experiments were conducted at a time interval found most convenient for each subject. A highly

significant relationship ($r = 0.795$) was found between age and time required to reach the peak chylomicron count. The regression line for these two variables is shown in figure 2. It, therefore, appears that the pattern of fat absorption changes with increasing age. Since it may be assumed that hydrolyzed fat does not contribute to blood chylomicrons, the absorption of relatively large amounts of neutral fat rather than products of fat hydrolysis occurs in older individuals.

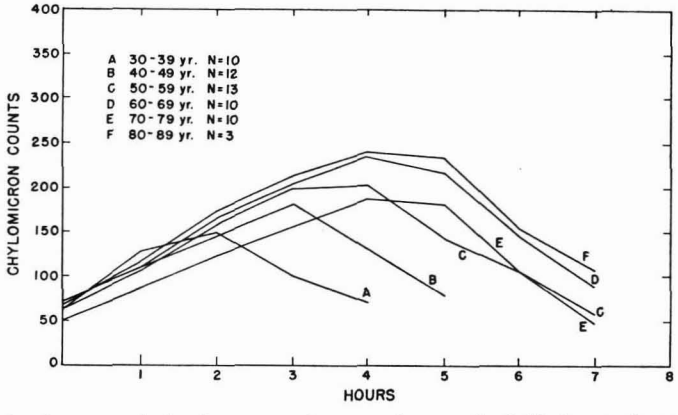

Fig. 1 Average chylomicron counts per microscopic field for each age-decade at hourly intervals following a test meal containing fat. N = number of subjects.

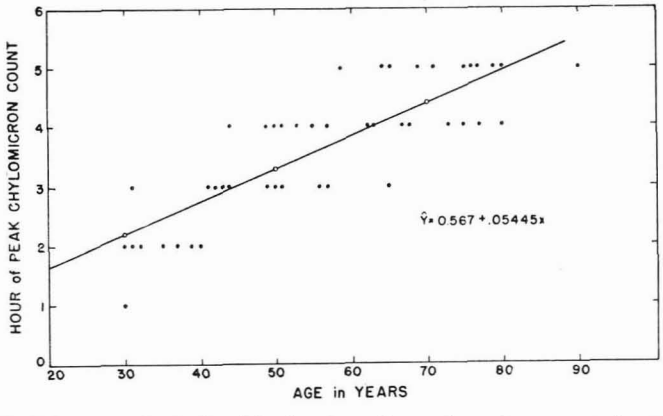

Fig. 2 Time required for blood of women of various ages to contain a maximum number of chylomicrons after a standard test meal.

Becker and his co-workers ('50) believe that either a decrease in pancreatic lipase or a low emulsifying power in the intestines may explain alterations in the fat absorption process in older people. There is also a possibility that the mechanism for the utilization of fat is altered with advancing years, so that a delayed removal of fat from the blood stream results.

Higher maximum numbers of chylomicrons were obtained in older women than in the younger subjects. The relationship between age and peak concentration of chylomicrons was highly significant ($r = 0.421$). The regression line is shown in

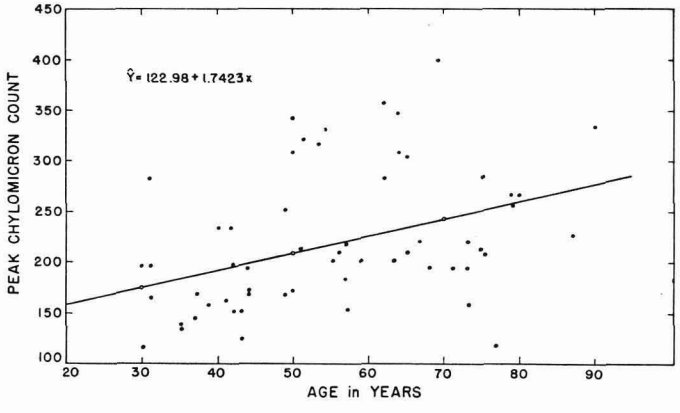

Fig. 3 The maximum number of chylomicrons in the blood after a standard test meal is eaten by subjects of different ages.

figure 3. If it is assumed that smaller numbers of chylomicrons in the blood stream are desirable, the low intake of fat frequently recommended for older people has a physiological basis. Furthermore, an increased incidence in atherosclerosis has been associated with high fat diets by some workers (Moreton, '47; Necheles, '51; Keys, '52).

The data next were rearranged into groups which reached a peak chylomicron count at the same time interval after the test meal, and the respective chylomicron curves drawn (fig. 4). The maximum chylomicron values for each group appeared to increase as the time required to reach the peak increased.

The average peak chylomicron count for each decade, regardless of the time at which it occurred after the test meal, is given in table 1. The peak chylomicron counts showed a steady increase up to the 7th decade followed by a drop in value.

A possible relationship between overweight and peak chylomicron count was considered in evaluating the present data. The weight of women at age 30 was taken as their ideal weight (Sherman, '47). Statistical treatment of the data showed no significant relationship between per cent of ideal weight and

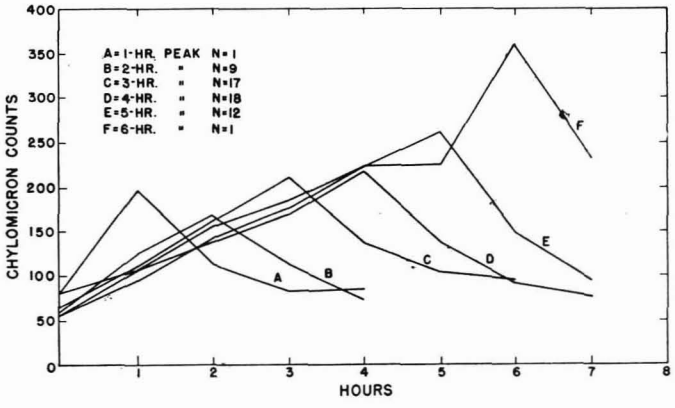

Fig. 4 Average concentration of chylomicrons in the blood of women who have been grouped according to the time required to attain a maximum count after a standard test meal. N = number of subjects.

the maximum number of chylomicrons. Recent findings by Walker ('53) indicate that weight loss is associated with a significant reduction in certain serum lipoprotein fractions (Sf 12–20, Sf 21–35 and Sf 35–100).

Serum cholesterol levels obtained in this study were related to age. This observation agrees with previous findings from this laboratory for a similar population (Swanson et al., '55). Table 1 summarizes the average serum cholesterol concentrations in each decade. Increasing serum cholesterol values were noted with advancing age up to the 7th decade, followed by a decline in later years. Although a similar trend was noted with

the peak chylomicron counts, the relationship between individual serum cholesterol levels and peak chylomicron counts was significant only at the 10% level.

TABLE 1

Average peak chylomicron counts, irrespective of time required to attain them, and average serum cholesterol levels for each age-decade

DECADE	NUMBER OF SUBJECTS	PEAK CHYLOMICRON COUNTS	SERUM CHOLESTEROL
			mg/100 ml
30–39	10	162 ± 26.8 [1]	246 ± 54.2 [1]
40–49	12	186 ± 38.2	236 ± 44.7
50–59	13	241 ± 63.4	284 ± 43.6
60–69	10	283 ± 72.6	320 ± 60.9
70–79	10	209 ± 46.6	285 ± 61.5
80–90	3	277 ± 52.5	275 ± 5.1

[1] Standard deviation.

SUMMARY

1. The pattern of fat absorption changes with advancing age.

2. The maximum number of chylomicrons appearing in the blood after a standard test meal increases with advancing age.

3. The longer the period required to reach a peak count, the greater the number of chylomicrons in the blood at the time.

4. Concentrations of serum cholesterol increase with age up to the 7th decade when a decrease occurs. The peak chylomicron count following a high fat test meal shows the same trend, but no statistically significant relationship was found between serum cholesterol concentrations and the peak chylomicron count. Thus, serum cholesterol may not be related to the pattern of fat absorption.

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HEMOGLOBIN CONCENTRATION, THYROID WEIGHT AND GROWTH RATE IN RATS DURING MINIMUM FLUORIDE INGESTION [1]

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The caries-preventive effects of fluoride ingestion during tooth development have been widely recognized in man and in experimental animals (Moulton, '46; Shaw, '54). This demonstrated means to increase the future caries-resistance of developing teeth is a strong argument for precision fluoridation of public water supplies at optimum levels providing that there are no concommitant harmful effects. On the basis of detailed surveys among human populations where there has been prolonged consumption of waters containing low levels of fluorides, there is a lack of incriminating evidence of any systemic toxicity, other than the formation of mottled teeth when the water contained in excess of about 2.5 ppm of fluorides.

Various isolated items of controversial nature from laboratory investigations exist in the literature about the toxicity and biological activity of the fluorides when ingested over prolonged periods. Adverse effects on hemoglobin formation, growth and development, thyroid physiology, reproductive ability and longevity have been suggested as manifestations of the prolonged ingestion of low levels of fluorides. In view of the potential importance of fluoridation to human health, it was considered desirable to investigate the effect of pro-

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longed ingestion of minimum amounts of fluorides by the laboratory rat on the rate of growth, rate of hemoglobin formation, thyroid size, bone fluoride storage, reproductive ability and longevity.

EXPERIMENTAL

Ninety-three weanling rats representing 12 litters were distributed into 8 experimental groups. Two strains of rats were employed, Holtzman and Long-Evans. Each experimental group consisted of 6 male rats and 5 or 6 females. The experiment was continued for a total of 11 months. During this time the rats were fed either one of two cariogenic diets. The rats in groups 1 through 4 received a purified ration of the type routinely used in our dental caries studies (Shaw, '47). The rats in groups 5 through 8 received a diet which consisted largely of natural foodstuffs in the following percentages: whole wheat flour 27, yellow cornmeal 26.5, whole milk powder 30, corn oil with added vitamins A, D, E, and K 5, crude casein with added water-soluble vitamins 9, salt mixture 2, and sodium chloride 0.5. The vitamin-enriched corn oil and casein, and the salt mixture were the same materials as used in the purified ration (Shaw, '47). Various batches of the two diets contained from 1.0 to 1.4 ppm of fluoride. No attempt was made to obtain materials which contained lower amounts of fluorides than those routinely available.

The rats in groups 1 and 5 received distilled water. Those in groups 2 and 6 received distilled water with enough sodium fluoride added to give a concentration of 1 ppm of fluoride. Sufficient sodium fluoride was added to the water for groups 3 and 7 to provide 5 ppm of fluoride and for groups 4 and 8 to give 20 ppm of fluoride. All rats were individually housed in screen-bottom cages. Food and water were provided ad libitum.

During the first 5 experimental months, the rats were weighed weekly and bled every other week. The rats were wrapped in gauze, and the tip of the tail cut off. No anesthesia was necessary. Each time 20 µl of blood was with-

drawn from the tail for hemoglobin determination. A standard colorimetric method was used for the determination of hemoglobin. During the 6th and 7th experimental months, pairs of rats were mated in each experimental group.

After 11 experimental months, the animals were sacrificed, their heads preserved for caries evaluation and the humeri and femora saved for fluoride determination. Also the thyroid glands were weighed and preserved in normal sodium hydroxide for fluoride determination. Fluoride distillations were done by the method of Singer and Armstrong ('54); determinations of the amount of fluoride were made colorimetrically by the method of Icken and Blank ('53).

RESULTS

The rates of growth and the final weights were comparable in all experimental groups. Average weights at the end of the 4th experimental month are given in table 1. Neither diet and none of the levels of fluoride altered the rate of growth nor the final weights attained.

During the 4th and 5th experimental months, several animals in each group died due to a severe respiratory infection which reached epidemic proportions during the hot and humid summer months of August and September 1953. The number of animals lost in groups 1 through 8 was 4, 4, 2, 3, 5, 3, 2, and 4 respectively. This death distribution indicates that the addition of varying amounts of fluoride had not altered the resistance of these rats to withstand this type of infection.

Hemoglobin values were low in all experimental rats at weaning, averaging 10 gm per 100 ml of blood, increasing to 12.5 gm within one week and reaching 15 gm after one month, regardless of whether fluoride was added or not. Repeated values taken over the first 140 days are presented in table 1.

Some depression in hemoglobin was observed during the hot summer months, which coincided also with the deaths of some animals from the respiratory infection. However, these decreases were universal through all groups and were independent of the level of ingested fluoride. At the end of the

TABLE 1

Weight and hemoglobin responses in rats during ingestion of varying amounts of fluoride

GROUP NO.	F CONC. IN WATER	AV. WT. ON 120TH DAY		HEMOGLOBIN CONCENTRATION IN GRAMS DURING EXPERIMENT								
				Days on experiment								
		♂	♀	1	8	15	21	28	38	65	94	140
	ppm	*gm*	*gm*									
1	0	367	243	9.1	12.6	12.9	13.5	13.4	14.9	15.4	14.3	14.6
2	1	344	236	9.8	12.8	13.4	13.1	13.8	15.4	15.2	14.8	15.3
3	5	367	235	10.0	12.2	12.9	13.2	14.0	14.6	15.5	15.1	15.0
4	20	363	242	10.3	12.1	12.7	13.7	13.9	14.8	15.6	14.1	14.8
5	0	360	228	9.5	12.1	12.2	13.0	13.9	14.7	15.6	13.8	14.4
6	1	390	222	10.0	12.1	12.7	13.5	14.2	15.1	15.1	14.8	15.1
7	5	375	240	10.2	12.9	12.6	13.3	14.0	14.5	15.5	14.4	14.7
8	20	410	237	10.1	12.2	12.4	12.9	13.4	14.9	15.1	14.4	14.5

5th experimental month hemoglobin determinations were discontinued as there was no trend toward a difference in any of the groups.

At this time pairs of rats were mated in each experimental group. At least two litters were born in each of the 8 groups representing the different fluoride levels. The litters were of normal size with no evidence of any increases in resorptions or still births among the fluoride groups. In addition, all of the rats born were normal, healthy offspring which grew and developed satisfactorily. They were sacrificed soon after weaning with no evidence of any structural abnormality.

Upon the termination of this experiment, the jaws were evaluated for carious lesions; the dental caries experiences of the 8 groups are given in table 2. Highly significant reductions were observed in the number of carious molars, the number of carious lesions and the extent of carious lesions in the two groups which received 20 ppm of fluoride in the drinking water. No significant reductions in caries experience were observed in groups which received 1 and 5 ppm of fluoride. This is in keeping with other experiments where fluorides have been given in the drinking water after tooth development has been largely completed.

The fluoride uptake in the skeletal system was determined in each group by analyses on the femora and humeri. The values are presented in table 2. Progressive increases in fluoride content were observed with increased ingestion of fluoride. Comparable amounts were stored in the bones of rats fed the purified and natural diets. These values are consistent with the wealth of data available in the literature on this subject.

No weight differences in the thyroid glands were observed with varying fluoride intake (table 2). Fluoride determinations were done on pooled thyroid samples, but no fluoride could be detected by the method used in our laboratory, as there was less than 1 μg of fluoride per three thyroid glands (50 mg) in the groups which received 20 ppm of fluoride.

TABLE 2

Comparison of bone fluoride content, thyroid weight and dental caries experience in rats which received varying amounts of water-borne fluorides

GROUP	DIET	F CONC. IN WATER	BONE F CONTENT	AVERAGE THYROID WEIGHT	NUMBER OF CARIOUS MOLARS			NUMBER OF CARIOUS LESIONS			EXTENT OF CARIOUS LESIONS		
					Av.	SEM [1]	CR [2]	Av.	SEM [1]	CR [2]	Av.	SEM [1]	CR [2]
		ppm	*ppm*	*mg*									
1		0	41 ± 5	18.8	2.5	0.3		3.5	0.6		10.9 +	2.6 +	
							0.1			0.6			0.5
2		1	145 ± 32	19.3	2.4	0.5		3.0	0.6		9.4 +	1.8 +	
	100						0.8			1.3			1.1
3		5	417 ± 57	18.6	2.1	0.4		2.6	0.4		7.7 +	1.1 +	
							3.8			3.6			3.1
4		20	1857 ± 177	19.1	0.9	0.3		0.9	0.4		2.2 +	1.0 +	
5		0	25 ± 0.3	19.2	3.1	0.4		4.6	0.7		18.1 +	2.8 +	
							0.0			0.3			0.6
6		1	121 ± 12	23.6	3.1	0.5		4.9	0.7		20.7 +	3.6 +	
	Nat.						0.9			0.8			1.3
7		5	503 ± 36	18.6	2.6	0.4		3.9	0.5		13.3 +	1.6 +	
							4.2			4.3			4.1
8		20	1669 ± 169	17.7	1.0	0.3		1.2	0.4		4.5 +	1.8 +	

[1] Standard error of mean.

[2] Critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the ratio is 3.0 or higher, the difference is highly significant.

DISCUSSION

The above results indicate that the levels of fluoride used in the course of the 11-month period of the experiment did not adversely affect the rate of growth nor the final body weight, the rate of hemoglobin formation, the thyroid size, the reproductive ability nor the longevity of the experimental subjects. For reasons which are presently unknown, these results differ somewhat from isolated data presented elsewhere.

For example, Matt ('53, '54) reported that the administration of minimum amounts of water-borne fluorides to recently weaned rats caused a temporary decrease in hemoglobin level that was roughly proportional to the amount of fluoride. The average hemoglobin levels in the rats that received 0, 1, 5, and 15 ppm were 15.8, 15.1, 13.6 and 13.5 gm/100 ml of blood, respectively, on the 30th experimental day. The comparable values on the 60th day were 16.2, 16.1, 15.7 and 15.5 gm, respectively. Body weights were slightly lower in the groups which received 5 and 15 ppm of fluoride in the drinking water. No comments were made as to any possible interfering situations which might have influenced the results. Likewise the fluoride content of the diet was not mentioned. At the higher levels of administration of 1 to 2 mg of sodium fluoride per kilogram of body weight, Risi ('31) observed a reduction in the number of erythrocytes in dogs. Reductions in erythrocyte count by 1 to 3 million were noted from the initial level of 7 to 8 million. This represents approximately a 15- to 30-fold increase in fluoride intake over the level believed to be optimum for growing children. Valjavec ('32) observed a tendency in rabbits for the hemoglobin concentration and the erythrocyte count to decrease after the intravenous injection of the overwhelming dose of 50 to 60 mg of sodium fluoride per kilogram of body weight. Only the levels of fluoride used by Matt ('53, '54) appear to be comparable to those in the present experiment. If the basal diet used in Matt's studies was relatively high in fluorides as is frequently the case in common brands of laboratory chow, the fluoride ingestion of his rats may have exceeded that of

our experimental subjects. In any case, there is a difference in the hemoglobin results between the two experiments. It is interesting to note that the rats in our experimental groups had uniformly low hemoglobin values at the beginning of the study. In the course of the first weeks, these values were increased to the normal range independent of the fluoride content of the drinking water. Thus in spite of this initial penalty, fluoride ingestion did not interfere with hemoglobin formation.

An inhibition of growth has been observed in pair-fed rats when 313 ppm of fluorine as sodium or calcium fluoride were added to the diet (McClure and Mitchell, '31). Sharpless ('36) observed that growth was unimpaired when rats were fed 250 ppm of sodium fluoride but was slowed by one-third by the ingestion of 1000 ppm. Since the amount of fluorides in the present experiment is far below these levels, it is not surprising that no influence upon growth was observed.

Effects of fluoride ingestion on human reproductive ability have been intimated in the press and in public forums by opponents of water fluoridation but even upon the addition of 226 ppm of fluoride to the ration Smith and Leverton ('33) were able to raise 4 generations of rats. The young were smaller at birth at this dosage level. Reproduction failed only when 452 ppm of fluoride were added. In contrast, Mick ('53) reported that there were numerous congenital abnormalities in the offspring of female rats fed what was described in some places as 1 ppm and in others as 2 ppm of fluorides in the drinking water throughout pregnancy. No evidence of any such abnormalities was observed in the present experiment when 1, 5 and 20 ppm of fluorides were ingested in the drinking water.

In the present experiment, no influence of fluoride ingestion was noted in the thyroid. However, Gordonoff and Minder ('53) reported that through increased fluoride intake, there was an increased fluorine and decreased iodine uptake by the thyroid. They postulated that this gland could not differen-

tiate between these two halogens and expressed doubts about the advisability of water fluoridation in areas of endemic goiter. However, in a study in which the differences in caries incidence were small and possibly of questionable significance, Muhler and Shafer ('54) presented evidence which was somewhat suggestive of a supplementary activity between fluorides and the thyroid gland with respect to dental caries. They found that desiccated thyroid (10 to 60 mg per day depending upon the growth of the animal) reduced the incidence of dental caries to the same degree as 20 ppm of fluoride in the drinking water. The ingestion of fluorides and desiccated thyroid combined resulted in a reduction that was approximately the sum of the reductions caused by the two separately. They postulated that the caries-preventive activity of fluorides might be partially mediated through the thyroid by stimulating the salivary flow as an end result of an increased basal metabolic rate. Mention was not made of the possibility that the two agents could have operated independently of each other.

The rate of fatalities was unusually high due to the severe respiratory infection which occurred in our rat population during this experiment. No difference in rate of fatalities was observed between the high and low fluoride groups. Few fluoride experiments have been conducted for sufficiently long periods to evaluate the influence upon longevity. A series of experiments with cancer-susceptible mice has been reported by Taylor ('54) in which a 9 to 10% shorter survival period was noted among the mice fed 1 or 10 ppm of fluoride in the drinking water. There are two interesting and perplexing observations in these results. First, the groups which received 10 ppm had comparable survival rates to those who received 1 ppm. Second, mice fed laboratory chow with a fluoride content varying from 20 to 38 ppm had the same survival rate as mice fed a natural diet containing a "negligible" amount of fluorides. This difference in results is explained in part by the author's statement that the availability of the fluorides present in laboratory chow is unknown. These com-

ments suggest that the data reported by Taylor were not of real significance or that there was a further variable that was not detected.

SUMMARY

When fluoride was administered in the drinking water to rats as sodium fluoride at levels of 1, 5 and 20 ppm, there was normal growth and reproductivity, no depression of hemoglobin formation, no detectable fluoride uptake by thyroid glands, no hypertrophic changes in the thyroid and no decrease in animal resistance to a severe respiratory infectious disease.

Dental caries incidence was significantly reduced when 20 ppm of fluoride were ingested in the drinking water.

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NIACIN AND ANTI-NIACIN ACTIVITY OF 3-ACETYLPYRIDINE IN DOGS [1]

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TWO FIGURES

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Woolley et al. ('38) observed symptoms of toxicity following oral administration of 192 mg of 3-acetylpyridine (3-AP) to dogs with blacktongue, but found the compound to be nontoxic in normal dogs when fed at the same level. Later Woolley ('45) reported that mice fed two or more milligrams of 3-AP per day developed symptoms resembling niacin deficiency which could be prevented by niacin or niacinamide (NA) or by tryptophan (Woolley, '46). Ackermann and Taylor ('48) reported inhibition of development in chick embryos injected with 3-AP which could be completely reversed by NA and to a lesser extent by niacin and by tryptophan. Gaebler and Beher ('51) reported marked increases in urinary N^1-methylnicotinamide (NMN) in both normal and niacin-depleted dogs following oral administration of 0.5 gm per day of 3-AP. No deleterious effects were observed in the normal dogs but manifestations of blacktongue as well as disturbances not reversible by niacin were observed in one niacin-depleted dog. After feeding C^{13}-labeled 3-AP to dogs and rats Beher et al. ('51, '52) concluded that the analogue was biologically oxidized to niacin to an extent sufficient to

A preliminary report of this work has been published (McDaniel, '53).

[2] The data presented herein were taken, in part, from a thesis submitted by E. G. McDaniel to the Graduate School of the George Washington University in partial fulfillment of the requirements for the degree of Master of Science.

account for the increased NMN observed following its ingestion. Beher and Anthony ('53) reported that between 20 and 30% of injected 3-AP could be detoxified by oxidation to niacin, and Beher et al. ('53) found that canine blood cells, liver and kidney were sites of detoxification.

In view of the disagreement in the literature as to the niacin or anti-niacin activity of 3-AP, the present series of experiments was done to determine more clearly the nature of the activity of this compound, both in normal dogs and during the development and treatment of niacin deficiency. Data are presented which demonstrate that the compound does possess niacin-like activity, but that under certain conditions the same compound also produces marked effects resembling the action of a niacin antagonist.

EXPERIMENTAL

Male and female mongrel dogs of various ages and sizes were housed in individual cages. Water and food were supplied ad libitum except to animals from which urine was to be collected, in which case food was usually offered for about two hours each day. Food consumption was recorded daily. Animals were weighed at least two times a week. The compositions of the diets are shown in table 1. Diet 123 is a corn diet similar to the blacktongue-producing diets used earlier in this laboratory (Sebrell et al., '37), and diet 9049 is a purified type ration similar to that used by Ruegamer et al. ('48).

Twenty-four-hour urine samples were collected in glass bottles containing 10 ml of 10% sulfuric acid, the volume measured, and a suitable aliquot stored at 4°C. until used. The method for the determination of urinary NMN was essentially that of Huff and Perlzweig ('47).

The 3-AP [3] used in these experiments was prepared in this laboratory by a procedure (Kolloff and Hunter, '41) which minimized the possibility of niacin as a contaminant. Niacin activity of samples tested was found to range from 1 part in

[3] The hydrochloride of 3-AP was used throughout these experiments. When amounts are indicated 3-AP HCl is implied.

400 to 1 part in 1000 as determined by *L. Arabinosis* assay (Snell and Wright, '41). NA and 3-AP were administered subcutaneously in aqueous solution, or orally by capsule as indicated. 3-AP, when given parenterally, was first neutralized with sodium bicarbonate.

TABLE 1

Composition of diets

CONSTITUENTS	DIET	
	123 [1,2]	9049 [3]
	%	%
Yellow corn meal	66.1	. . .
Cow peas	8.3	. . .
Casein (Vitamin-free)	10.0	18.0
Sucrose	5.3	67.0
Cottonseed oil (Wesson)	5.0	11.0
Cod liver oil	1.7	. . .
Salts (Osborne and Mendel)[4]	3.6	4.0

[1] To prepare diet 123, the corn meal, cow peas and salts were mixed with water and cooked in a double boiler 1½ hours. The casein, sucrose, cotton seed oil and cod liver oil were then stirred in with sufficient water to make a cooked weight of 400 gm per 100 gm of above ingredients.

[2] During the initial phase of this study symptoms of acute riboflavin deficiency were observed in one dog on diet 123. Riboflavin was then given to dogs on this diet as an oral supplement at a level of 0.1 mg per kilogram of body weight per day, or by incorporation into the diet at a level of 0.4 mg %.

[3] Dogs on diet 9049 were given, in gelatin capsules, a vitamin supplement which supplied the equivalent of 0.1 mg each of thiamine hydrochloride and riboflavin, and 0.6, 0.5 and 25 mg respectively of pyridoxine hydrochloride, calcium pantothenate and choline chloride per kilogram of body weight per day, and 500 I.U. vitamin D and 2500 I.U. vitamin A per day.

[4] Wesson modification of Osborne and Mendel salts was used during part of the experiments.

RESULTS

Effect of 3-AP upon urinary NMN

The quantitative relationship of 3-AP to niacin in normal dogs was explored by comparing NMN excretion of 8 dogs fed a commercial stock ration with and without known amounts of 3-AP and NA as indicated in table 2. Increases were observed following the administration of either 3-AP

TABLE 2

Average increases in daily urinary N'-methylnicotinamide (NMN) following administration of niacinamide (NA) and 3-acetylpyridine (3-AP) to normal stock-fed dogs[1]

DOG NO.	NORMAL	INCREASES IN DAILY URINARY NMN		INCREASE IN NMN PER 100 MG OF	
		After 100 mg NA	After 130 mg 3-AP	NA	3-AP
	mg	*mg*	*mg*	*mg*	*mg*
72	10.7	65.8 (oral)	27.9 (subcut.)	55	13
73	10.9	89.5 (subcut.)	28.1 (oral)	79	13
74	10.9		24.0 (subcut.)		10
75	15.5	56.8 (oral 50 mg)[2]	31.5 (subcut.)	83	12
76	13.4	96.6 (subcut.)	16.8 (oral 65 mg)[2]	83	5
77	11.3		26.2 (subcut.)		11
81	7.4		9.0 (subcut. 30 mg)[2]		5
117	2.7		4.7 (subcut. 30 mg)[2]		7

The stock diet used in these experiments was Purina Dog Chow.

[1] Each normal value represents the average of at least 10 daily determinations without administration of NA or 3-AP. Each experimental value represents the average of three or more separate tests. To avoid an accumulative effect at least one day without supplementation was allowed, after each test dose, during which the metabolite excretion returned to within the normal range.

Of the 4 dogs which received both NA and 3-AP, two received the NA prior to the 3-AP. The other two dogs received the 3-AP first.

[2] Smaller amounts administered to dogs 75 and 76 in oral tests, and to dogs 81 and 117.

or NA. On the basis of these increases it was estimated that in normal dogs an average of 8 mg (6 to 15 mg) of 3-AP are equivalent to 1 mg NA, or an activity of 1/6 on a molar basis.

A comparison was made of urinary NMN following administration of 3-AP and NA in niacin-deficient dogs (with and without blacktongue).[4] Two dogs (fig. 1), having received the niacin-deficient corn diet for over 600 days but without signs of blacktongue at the time of the test, and two dogs (fig. 2) which had developed marked blacktongue on the purified diet were treated subcutaneously with 3-AP and NA as

[4] The symptoms accompanying the development of blacktongue as observed in these experiments included some or all of the following: Formation of red band-like lesions on the mucosa of the upper lip; diffuse reddening of the throat, cheeks, floor of the mouth, and margins of the tongue; pseudomembrane formation on lips and cheeks; diarrhea; anorexia; excessive salivation; presence of a characteristic foul odor in the mouth; weight loss.

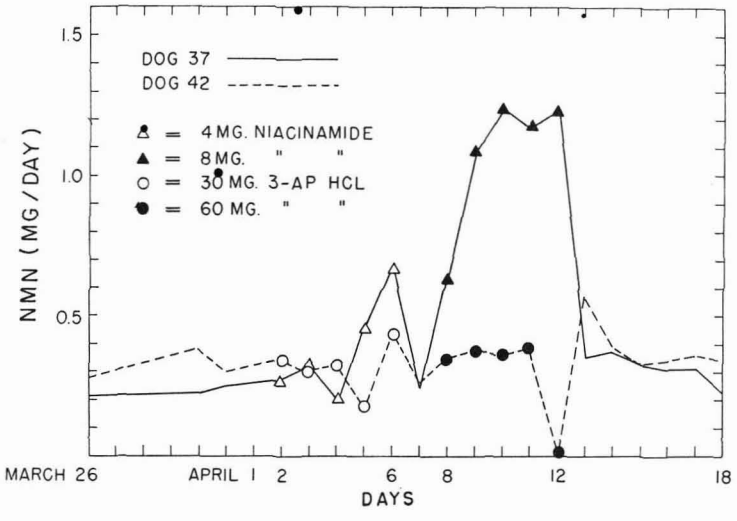

Fig. 1 Urinary N^1-methylnicotinamide (NMN) during administration of niacinamide (NA) and 3-acetylpyridine (3-AP) to niacin-deficient dogs without blacktongue.

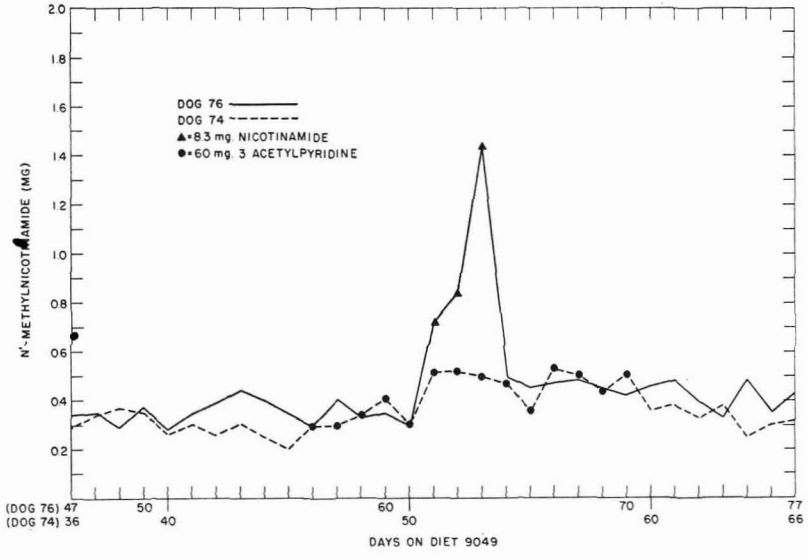

Fig. 2 Urinary N'-methylnicotinamide (NMN) during successful treatment of blacktongue with nicotinamide (NA) and 3-acetylpyridine (3-AP).

indicated. Only the administration of NA resulted in a marked increase in NMN. The symptoms of blacktongue disappeared following treatment with 3-AP or NA. During subsequent attacks of blacktongue in the two dogs fed the purified diet (fig. 2) treatment with 60 or 120 mg daily again failed to cause any marked increase in NMN. The larger dose of 3-AP did not increase urinary NMN detectably more than 60 mg. These results are in marked contrast to the NMN excretion following similar doses of 3-AP when these dogs were on a normal diet (table 2). Urinary NMN was followed on two additional dogs fed the purified diet during treatment of blacktongue with 3-AP. One was treated subcutaneously with 30 mg of 3-AP per day for 18 days, and although the blacktongue was cured there was no significant increase in the level of NMN excreted. The urinary NMN for the other dog increased from a pretreatment level of 0.2 mg per day to 0.44 mg per day following treatment with 30 mg of 3-AP daily for 26 days, then gradually to above 4.0 mg per day after the daily injection of 3-AP had been increased to 60 mg. This was the only instance in 6 tests with 3-AP in niacin-deficient dogs where a substantial increase in urinary NMN was observed. As shown in table 2, administration of 3-AP to 8 normal dogs led to substantial increases in NMN in every instance.

The above results indicated that although the normal dog was able to convert 3-AP to niacin (6 units of 3-AP $\simeq$ 1 unit of NA) the ability of the niacin-deficient dog to make this conversion appeared to be greatly decreased. As a further check on this point urinary NMN was followed in 6 dogs (litter mates) from the time of starting the deficient diets. Three dogs received the corn diet and three the purified diet. One dog on each diet was given daily a subcutaneous injection of 60 mg of 3-AP. NMN excretion, initially 11 to 16 mg per day, declined rapidly in all dogs. In dogs which received 3-AP this decrease was less rapid, becoming relatively constant at 3 to 4 mg per day in 40 to 60 days. Dogs receiving the unsupplemented purified diet reached a level of approximately 0.5 mg per day in 30 days, whereas dogs receiving the

unsupplemented corn diet stabilized at about 1.0 mg per day at 40 days. (The corn diet contained 1.76 mg of niacin per 100 gm whereas the purified diet was virtually free of the vitamin.[5]) Although during the early part of the experiment the NMN values for the 3-AP-supplemented dogs were considerably higher than for the unsupplemented dogs, the levels gradually decreased until about 280 days when the NMN values for supplemented and unsupplemented dogs were not significantly different (about 0.3 mg per day). These results suggested that dogs may lose their ability to convert 3-AP to niacin as they subsist for long periods on the deficient diets.

Blacktongue prevention with 3-AP

Seven dogs were fed the corn diet of which 6 received oral supplements of 260 mg and one received subcutaneous injections of 60 mg of 3-AP daily. Of the 6 dogs receiving 260 mg per day, two displayed symptoms of toxicity and died within 41 days. One showed somewhat less marked toxic symptoms which disappeared when 3-AP was discontinued after 51 days; blacktongue developed 119 days later. The three remaining dogs receiving 260 mg of 3-AP per day showed no symptoms of toxicity and failed to develop blacktongue within one year. 3-AP was then discontinued for one dog and blacktongue developed in 139 days. In the second dog 3-AP was reduced to 25 mg per day for 131 days without blacktongue, but the disease did develop 17 days after the drug was reduced to 0.2 mg per killogram of body weight per day. The third dog failed to develop blacktongue even though the 3-AP was reduced to 25 mg per day for 132 days, then to 0.2 mg per kilogram of body weight per day for 125 days, and finally discontinued completely for an additional 65 days. The dog receiving 60 mg of 3-AP per day was maintained for 467 days without symptoms of toxicity or deficiency, then developed blacktongue between 4 and 10 days after 3-AP was discontinued.

[5] We are indebted to Mr. Howard Bakerman of this Institute for the niacin assays.

The results obtained in these preventive experiments with the corn diet were not conclusive since one dog did not develop blacktongue after the 3-AP was discontinued, and two of three additional unsupplemented control dogs on this same diet behaved unexpectedly in that blacktongue did not develop in the usual time. One of these unsupplemented dogs did develop blacktongue in 287 days, but only after an oral supplement of 0.5 gm of indole had been given for 78 days.[6] The other dog did not develop blacktongue until the 543rd day.[7] The third control dog developed blacktongue in 93 days. In spite of this limitation the results suggested a preventive action of 3-AP, in that 4 of the dogs which had received preventive doses of the drug did develop blacktongue after the 3-AP was discontinued or reduced to a low level.

To evaluate more accurately any blacktongue-preventive action of 3-AP, the drug was tested on dogs fed the purified diet with which blacktongue was routinely produced in short periods. Ten dogs were fed the purified diet of which 6 received neither NA nor 3-AP and developed blacktongue in an average of 53 days (29 to 74 days). Three dogs were fed 260 mg of 3-AP per day of which two failed to tolerate the drug. One died in 9 days, the other lost weight rapidly and the 3-AP was discontinued after 60 days. Blacktongue developed in the latter dog 18 days after the 3-AP was stopped. The third dog showed no evidence of toxicity and failed to develop blacktongue in 138 days when death occurred because of massive intra-abdominal hemorrhage. One dog on the purified diet received 60 mg of 3-AP subcutaneously per day for 467 days without blacktongue, then developed the disease 29 days after the 3-AP was discontinued. Another dog (one of

[6] Indole seems to have the property of precipitating attacks of blacktongue (Sebrell and Hundley, '48; McDaniel, unpublished results).

[7] The diet of this dog was changed from the cooked to the uncooked form on the 260th day. Unpublished results from this laboratory indicate that the uncooked diet has a greater capacity to produce blacktongue than the cooked form. Also the supplementary riboflavin was discontinued after the 467th day although there is no assurance that this had any effect on the eventual development of blacktongue.

the 6 controls), which had developed blacktongue on the purified diet, was treated with 30 mg of 3-AP subcutaneously for 26 days and thereafter with 60 mg daily for 272 days without blacktongue. The disease did develop 35 days after 3-AP was stopped.

Treatment of blacktongue with 3-AP

One dog on the corn diet which developed blacktongue when the daily dose of 3-AP was reduced to 0.2 mg per kilogram body weight, was treated with 25 mg of 3-AP daily by mouth. All symptoms of blacktongue disappeared in 11 days. This same dog was allowed to develop a second attack of blacktongue. Again, an oral supplement of 25 mg of 3-AP daily resulted in a cure in 12 days (although toxic effects terminating in death developed). A second dog in which blacktongue had developed after 93 days on the corn diet, was cured with 25 mg of 3-AP per day orally for 12 days. A second attack of blacktongue developed 8 days after the 3-AP was stopped and this attack was also cured with similar treatment for 11 days. This is the only instance in which two consecutive attacks of blacktongue have been cured with 3-AP without the appearance of toxic symptoms.

Six dogs on the purified diet were allowed to develop blacktongue for treatment with 3-AP. Of 4 dogs treated subcutaneously with 30 mg of 3-AP per day, one died within 24 hours after the first injection, and in the remaining three dogs the symptoms of blacktongue were cured in 4 to 8 days. Two dogs were treated subcutaneously with 60 mg of 3-AP per day. Blacktongue was cured in 6 days in one dog, the other showed definite improvement in the blacktongue lesions, but developed anorexia, lost weight rapidly and died after 9 days of treatment. The first of these two was treated with 120 mg of 3-AP per day during a second attack of blacktongue and in spite of some improvement in the blacktongue, toxic symptoms developed which ended in death.

The above results indicate that 3-AP can cure blacktongue when given in moderate amounts. In 11 attempts with 25 to 60 mg of 3-AP per day complete cure of the symptoms of the disease was observed in 8 cases, some improvement was noted in two cases, and one animal died after the first injection. Toxic symptoms were observed in some of the dogs even though blacktongue was cured. In 4 attempts to treat two consecutive attacks of blacktongue with 3-AP, only one attempt has been accomplished without the development of toxic symptoms which terminated in death during the second treatment.

Toxicity of 3-AP

A marked difference was noted in the toxicity of 3-AP between dogs which were deficient in niacin and those which were not. Dogs on the stock diet tolerated 260 to 520 mg of 3-AP (oral) for three to 5 days with no evidence of toxicity. Dogs on the niacin-deficient diets but relatively well nourished with respect to niacin could tolerate daily doses of 60 to 120 mg of 3-AP over long periods without evidence of toxicity. Daily doses of 260 mg were tolerated by about half of the dogs for periods of 138 days to more than a year without toxicity whereas the remainder received the drug for 9 to 51 days before death or marked toxic symptoms forced a discontinuation of the tests.

These results are in striking contrast to that in dogs with or without blacktongue which were obviously deficient in niacin. On the corn diet, two dogs which had previously tolerated doses of 260 mg of 3-AP for 51 and 364 days, respectively, died within 5 hours after single oral doses of 260 mg of 3-AP given as treatment for mild blacktongue. One dog which had received previously 260 mg of 3-AP daily for more than one year, was cured of blacktongue with 25 mg of 3-AP daily (oral) without evidence of toxicity; however during the next attack of blacktongue this dog developed marked toxic symptoms although the blacktongue was cured with the same dose of the drug. Another dog on the corn diet

with marked blacktongue died within 24 hours after a single injection of 30 mg of 3-AP. With the purified diet 9 attacks of niacin deficiency were treated with 30 to 120 mg of 3-AP daily. Three dogs were cured of blacktongue without toxicity (all first episodes of blacktongue), one died within 24 hours after the first injection of 30 mg, the remaining 5 developed marked symptoms of toxicity resulting in death in each instance.

There was also a marked difference in the toxicity symptoms observed. Dogs not suffering from niacin deficiency showed only gradually developing anorexia, weight loss and sometimes early death. On the other hand dogs with definite niacin deficiency exhibited marked symptoms such as paralysis of the tongue, eyelids and legs (most marked in hind legs), tremors, weakness, nervousness, excessive salivation, thick ropy secretion from the eyes, anorexia and weight loss. In all cases after these toxic symptoms developed, the condition appeared to be irreversible and terminated in death, even when the drug was stopped and large doses of NA were given.

Prevention of toxicity with niacinamide

One test was made to determine whether NA could prevent the toxic effects of 3-AP in deficient dogs. Two dogs on the purified diet, judged to be about equally deficient in niacin, were used. Both were given 100 mg of 3-AP subcutaneously the first day, then 200 mg on each of the subsequent days. One of these received 100 mg of NA intravenously just prior to each of 7 daily injections of 3-AP. No symptoms of toxicity appeared, the blacktongue lesions disappeared, appetite and body weight improved and the animal appeared normal. The second dog developed marked toxic symptoms after the second dose of 3-AP and died in 7 days despite discontinuance of 3-AP after 5 injections and the subcutaneous administration of 100 mg of niacinamide daily beginning the day after toxic symptoms appeared.

DISCUSSION

It is clear that 3-AP can serve to replace dietary niacin as shown by the facts that administration of the compound leads to the urinary excretion of increased amounts of NMN and that it can prevent and cure blacktongue and other symptoms of niacin deficiency in dogs. Furthermore, Beher et al. ('52, '53) have proved the conversion of 3-AP to NMN using C^{13}-labeled 3-AP. The niacin-deficient dog appears to have a lesser capacity to transform 3-AP into NA as shown by the fact that normal dogs tolerate doses of 3-AP which are highly toxic to niacin-deficient animals. Furthermore, deficient dogs given 3-AP show little or no increase in urinary NMN whereas normal dogs given the same doses excrete large amounts. A part of this failure to excrete NMN may be due to the necessity to replete the stores of niacin in the tissues. This can hardly be the entire explanation however. Beher and Anthony ('53) estimate that dogs can convert 20 to 30% of 3-AP to niacin. Our results indicate a conversion in normal dogs of about 17% based on urinary NMN alone and making no allowance for other metabolites of niacin which are formed (as shown by Beher and Anthony, '53; and Rosen, '51). Since doses of 4 to 8 mg of NA produced definite increases in urinary NMN in deficient dogs whereas doses of 30 to 120 mg of 3-AP failed to do so (with one exception), a diminished power of conversion in deficient dogs seems indicated. Beher et al. ('53) have preliminary evidence that Coenzymes I and II may actually be involved in the process by which 3-AP is converted to niacin.

These facts may explain the increased toxicity of 3-AP in niacin-deficient dogs. Kaplan and Ciotti ('54) have shown that 3-AP can be exchanged for NA in Coenzyme I (DPN) by pig brain DPNase and that the resultant 3-AP DPN has much reduced enzymatic activity. Furthermore, by injecting 3-AP into mice they obtained evidence of 3-AP DPN in mouse brain. Thus, it is logical that the niacin-depleted dog with diminished tissue stores of DPN and TPN would be much more susceptible to 3-AP since it could ill afford to have any

of its niacin-containing coenzymes converted into "inactive" 3-AP forms and at the same time have a diminished capacity to convert 3-AP to NA to replenish its stocks of DPN and TPN. This double effect may explain the apparently irreversible nature of 3-AP toxicity observed in some of our dogs. These facts indicate that the 3-AP molecule is an antimetabolite of niacin.

Thus, it seems that 3-AP is an unique compound in that it has a dual role in niacin metabolism being a precursor of niacin on one hand and an antagonist on the other. Animals apparently have a certain capacity to transform 3-AP into niacin. When this capacity is exceeded, toxicity (antagonism) results. Whether all of its toxicity is due to its anti-metabolite function is not entirely clear. The fact that toxicity was completely prevented in one niacin-deficient dog pretreated with NA and the much greater tolerance of normal dogs for 3-AP suggests that the toxicity is due primarily to its antimetabolite function.

It is of some interest that this dual niacin, anti-niacin action of 3-AP is not confined to the dog. Experiments in this laboratory have shown a similar situation in rats (Hundley, '52). In niacin-deficient rats 65 mg % of 3-AP in the diet had approximately the same curative activity as 2 mg % of niacin. A single dose of 2.5 mg of 3-AP was an approximate LD 50 for 50 to 80 gm niacin-deficient rats whereas normal rats of similar size tolerated this and larger doses with no toxicity. Toxicity could be prevented completely with NA but not with niacin. Toxicity symptoms in rats were very similar to the marked toxicity symptoms described in deficient dogs.

Handler and Featherstone ('43) reported that animals carried through blacktongue with saline and into more advanced states of niacin deficiency displayed anemia as a prominent finding. In our experiments hematocrit, hemoglobin, erythrocyte and leucocyte determinations were made frequently. No abnormalities other than mild anemia were encountered in niacin-deficient dogs. If one regards the 3-AP toxicity syn-

drome as a very severe niacin deficiency, it is of interest that no abnormal blood findings other than that ordinarily found in blacktongue were encountered in 3-AP-toxic animals.

SUMMARY

3-AP has been shown to have both niacin and anti-niacin activity. It is about 1/6 as active as NA in increasing urinary excretion of NMN in normal dogs fed a stock diet. Twenty-five to 260 mg of 3-AP daily protected dogs against blacktongue over long periods. Daily doses of 25 to 60 mg of 3-AP were sufficient to cure blacktongue.

Niacin-deficient dogs appeared to have a diminished capacity to convert 3-AP to niacin as shown by urinary NMN excretion studies and by the fact that doses of 25 to 260 mg of 3-AP were highly toxic in deficient dogs but relatively well tolerated by normal dogs. NA prevented 3-AP toxicity but was ineffective once the toxic syndrome had developed.

The data suggest that animals have a limited capacity to transform 3-AP to niacin. When the dosage of 3-AP exceeds this capacity, the 3-AP molecule acts as a specific antagonist to niacin.

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EFFECT OF GENISTIN ON REPRODUCTION OF THE MOUSE[1,2]

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Reproductive disturbances have been reported to occur in sheep and in rabbits fed the soybean plant as a large part of the diet (Hunt, '35; Kendall et al., '50; Matrone, '52). That these reproductive disturbances might have been caused in part by the presence of an estrogenic-like substance can be inferred from data in the literature. The evidence is as follows: first, these reproductive disturbances are similar to those reported to occur in sheep grazing on the estrogenically active subterranean clover pastures in Australia (Bennetts and Underwood, '49; Underwood and Shier, '51); and second, the compound, genistein (4′ 5, 7-trihydroxyisoflavone), responsible for the estrogenic activity of subterranean clover (Bradbury and White, '51) also is present in soybean oil meal as the glucoside of genistein, genistin (Walz, '31; Walter, '41).

Injections of genistin have shown it to be estrogenically active (Cheng et al., '53). By means of the mouse uterine weight assay, Carter and associates ('53) have shown that commercial soybean oil meal also is estrogenically active but that commercial soybean oil meal residue from which genistin has been extracted is inactive. The present report, a part of

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[2] Supported in part by Tennessee Valley Authority, Knoxville, Tennessee.

an investigation of factors in the soybean plant that affect reproduction, deals with the dietary effect of soybean-genistin on the reproduction of the mouse.

EXPERIMENTAL

The dietary variables studied were soybean oil meal and the genistin extracted from commercial soybean oil meal. The diets formulated for this purpose are shown in table 1.

TABLE 1

Ingredients of test diets fed mice during growth and reproduction

CONSTITUENTS	AMOUNT PER KILOGRAM OF DIET		
	Diet I	Diet II	Diet III
	gm	*gm*	*gm*
Commercial soybean oil meal	....	....	800.0
Extracted soybean oil meal [1]	800.0	800.0	....
Glucose [2 3]	100.0	98.0	100.0
Wesson oil [4 5]	60.0	60.0	60.0
Mineral mix [6]	40.0	40.0	40.0
Genistin [7]	....	2.0	....

[1] Commercial soybean oil meal extracted with hot methanol.

[2] "Cerelose," Corn Products Refining Company, New York, N. Y.

[3] Water soluble vitamins and methionine added replaced an equal weight of glucose.

[4] Wesson Oil and Snowdrift Sales Company, New Orleans, La.

[5] Oleum percomorphum and alpha-tocopherol were dissolved in Wesson oil.

[6] Wesson's modification of the Osborne and Mendel salt mixture.

[7] Genistin isolated from commercial soybean oil meal.

Diet I was considered as the control since it was made up chiefly of soybean oil meal from which genistin had been extracted by Walter's method (Walter, '41). Meal thus extracted has been shown to be estrogenically inactive (Carter et al., '53). Diet II consisted of the same constituents as diet I except that genistin had been added to represent 0.2% of the diet. Diet III was made up chiefly of commercial soybean oil meal containing approximately 0.1% genistin (Walter, '41). Vitamins were added to provide, per kilogram of diet: 0.02 mg B_{12}, 0.2 mg biotin, 1 mg folic acid, 5 mg thiamine,

5 mg pyridoxine, 6 mg alpha-tocopherol acetate, 10 mg riboflavin, 10 mg nicotinic acid, 10 mg 2-methyl-1,4-naphthoquinone, 30 mg Ca-pantothenate, 1 gm para-aminobenzoic acid, 1 gm inositol, 1.5 gm choline chloride, and 24 drops of oleum percomorphum. The vitamins added to the diets were essentially the same as those added to synthetic diets for normal reproduction and lactation in the mouse (Fenton and Cowgill, '47), except for the addition of vitamin B_{12} and the substitution of oleum percomorphum for cod liver oil concentrate. In addition 6 gm of DL-methionine per kilogram of diet was added as a safety measure since soybeans are low in this amino acid. The salt mixture used was that devised by Wesson ('32).

The experiment, involving a total of 108 female Swiss albino mice, was a randomized block design in which the female mice were assigned to blocks on the basis of uniformity in weight. A block consisted of three cages to which the three experimental diets were assigned at random. Each cage housed three female mice.

In an effort to increase the probability of revealing any possible effect of dietary genistin and soybean oil meal on reproduction, female mice were placed on the experimental diets as soon as they were weaned, at approximately three weeks of age.[3] Observations on weight gain, feed intake, and time of vaginal opening were made over a period of 4 weeks, preliminary to the reproduction study. Since the time of the vaginal opening is indicative of estrogenic activity (Emmons, '50), this criterion was used as an index of the estrogenic activity of the diets.

The males used for breeding were raised on Purina Laboratory Chow. Following the growth study with the females, one male was assigned at random to each cage of females and left there for one 21-day period. The criteria for repro-

[3] Mice were furnished by M. P. Bailey of the North Carolina State Laboratory of Hygiene. Since no birth dates are kept at the State Laboratory of Hygiene, the mice are weaned according to weight. The weights of the mice selected for this study ranged from 9.4 to 12.1 gm.

duction were the number of young born alive per female on each diet, the number of litters dropped, and the number of young per litter.

RESULTS AND DISCUSSION

The average number of days from the time the females were placed on the experimental diets until the vaginas opened was: *Diet I (control) 16.3; diet II (genistin) 6.4; diet* III (commercial soybean oil meal) 5.9. An analysis of these data indicated that the difference in days required for vaginal opening was significantly higher ($P \leq 0.01$) for the mice fed diet I as compared to those fed diets II and III. A further evaluation of the estrogenic activity of the diets can be obtained by comparing the age at which vaginal openings occurred with the normal age of 35 days reported in the literature (Snell, '41). Since the mice in this experiment were approximately 21 days old when they were started on the diets, it is apparent that diet I is an adequate control because the vaginal opening of the mice on this diet occurred at a normal age. The mice on diets II and III, however, were less than 35 days old at the time of vaginal opening thus demonstrating the estrogenic activity of these diets.

The average food intake and weight gain, respectively, in grams per cage for the first 4-week period of the experiment were as follows: diet I, 253, 21.7; diet II, 256, 21.5; and diet III, 252, 21.2. An analysis of these data indicated no significant differences in either weight gains or food intake. The possibility remains, however, that genistin may have either beneficial or detrimental effects on growth under different conditions or at other levels.

The group average of the number of young born (per female on each diet), percentage of females dropping litters, average number of young per litter, and average weight per litter are presented in table 2. The group average of the number of young born for the control diet (diet I) was 4.9 and for the genistin diet (diet II), 3.2. A statistical analysis of these data indicated that the difference was significant

($P \leq 0.05$). On the per litter basis, the average for the mice on diet I (control) was 6.0, diet II (genistin) 5.4, diet III (commercial soybean oil meal) 5.8. Although the value for the genistin diet was lower than that of the other two diets, the difference was not significant, and all of the values were within the average range of 5 to 6 young per litter reported by Snell ('41). However, only 59% of the females fed diet II bore a litter, whereas 82% of the females fed diet I, and 77% of the females fed diet III bore litters. These data in-

TABLE 2

Summary of data from reproduction study

CRITERIA	DIET I [1] (control)	DIET II [1] (genistin)	DIET III [2] (commercial soybean oil meal)
Total no. of females	33	34	35
Group average of young born per female	4.9	3.2	4.5
Percentage of females dropping litters	82	59	77
Av. no. young per litter	6.0	5.4	5.8
Av. wt. per litter, gm	8.5	7.9	8.7

[1] Of 36 animals placed on diet one died during reproduction study, the remainder died during the growth study.

[2] One animal died during reproduction study.

dicate that the effect of genistin in the diet was on the number of litters dropped rather than on the number of young per litter.

Although the percentage of females bearing a litter was lower for the group on the soybean oil mean diet than either the reported normal for mice of 80 to 90% (Snell, '41), or the group of mice on the control diet, the difference was not marked enough to be considered significant. It is possible that the failure of the soybean oil meal diet (diet III) to give the same results as the genistin diet (diet II) was due to the fact that diet II contained more genistin than did diet III. This point deserves further investigation, particularly in

view of the fact that soybean oil meal *per se* has been shown to be estrogenically active (Carter et al., '53).

Clinical symptoms, such as dystocia, stillbirths and fetal resorption, which have been reported to occur in sheep on the estrogenically active subterranean clover pastures (Underwood, '52) and in rabbits on soybean hay (Kendall et al., '50) were not observed in this study with mice. The fact that the main effect of genistin was on the number of litters born and not on the size of the litter is further evidence that neither resorption nor intrauterine deaths occurred. Since a histological study of uteri, ovaries or other reproductive glands was not made, the reproductive disturbances cannot be attributed to the effect of genistin on any specific gland or tissue.

Since the males received the test diets during the 21-day period they were housed with the females, the possibility exists that the effect of genistin was mediated through the males. Each male, however, sired one or more litters, indicating that regardless of the dietary regime all males were fertile at some time, if not at all times, during the 21-day breeding period.

SUMMARY

A study was conducted to determine the effects of soybean oil meal and of genistin extracted from it on growth and reproduction of the mouse. Growth of the mice from three to 7 weeks of age was not affected by the treatments used in this experiment. Both commercial soybean oil meal and isolated genistin significantly lowered the age at which the vaginas of immature mice opened. The principal effect on reproduction of 0.2% genistin in the diet was a decrease in the number of litters born, whereas litter size was not affected. The effect of commercial soybean oil meal (80% of the diet) on the number of litters born was not statistically significant but the number of litters obtained was less than that from the group of females on the control diet.

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THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

XV. COMPARISON OF THE PROTECTIVE EFFECT OF LINOLEIC ACID AND LINOLENIC ACID AGAINST MULTIPLE SUBLETHAL DOSES OF X-IRRADIATION IN THE RAT [1,2]

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The fact that rats and mice fed diets containing fat are protected against radiation damage to a greater degree than animals fed fat-free diets is well established (Cheng et al., '52, '54a; Decker et al., '50; Ershoff, '54). The essential fatty acids present in the fat have been shown to be the effective factor in such protection. Thus, when a low-fat diet was supplemented with methyl linoleate in a dose as small as 10 mg per day, marked protection against x-irradiation was obtained. It was possible to extend the survival time of rats exposed to x-irradiation, over and above that of animals on the same low-fat regimen, when the former group was supplemented with linoleate (Deuel et al., '53). Later work in our laboratory (Cheng et al., '54a) demonstrated that the protective effect exerted on 20-week-old rats which were subjected to x-irradiation increased with increasing supplementary doses of methyl linoleate; better survival was noted when the daily dosage was 50 mg of linoleate as compared with 10 mg while a still greater protection was found in a group receiving 100 mg of this essential fatty acid per day.

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[2] Contribution no. 383 of the Department of Biochemistry and Nutrition, University of Southern California.

An earlier report from this laboratory (Greenberg et al., '50) showed that linolenate possessed a markedly lower potency in promoting growth, and in producing curative effects against essential fatty acid deficiency symptoms in rats, than did linoleate, unless it was "sparked" by the latter. These results were carried out with ordinary linolenic acid, 9,12,15-octadecatrienoic acid, which Thomasson ('53) has likewise found to have only slight biopotency against essential fatty acid deficiency. On the other hand, the latter investigator reported that γ-linolenic acid (6,9,12-octadecatrienoic acid) has a biological activity identical with that possessed by linoleic acid.

In the present experiments a comparison has been made of the ability of linolenate and linoleate to protect rats subjected to the stress of x-irradiation.

EXPERIMENTAL

Male rats of the University of Southern California strain were employed in these tests. They were placed at weaning on a fat-deficient diet similar to that employed by Cheng et al. ('54a). After a preliminary depletion period of 6 weeks, the animals were assigned to one of several groups; all animals continued to receive the basal fat-free diet. Supplements of ethyl laurate, methyl linoleate, or ethyl linolenate were administered, as indicated in table 1 and below, twice weekly *per os* using a 0.25 ml tuberculin syringe with a blunted needle. There were 13 or 14 rats in each group.

The several groups of rats received the following supplements twice weekly: group 1, negative control group, 0.25 ml of ethyl laurate [3] only; groups 2 to 5, 10, 20, 40, and 80 mg of methyl linoleate [4] per day respectively; groups 6 to 9, 10, 20, 40, and 80 mg of ethyl linolenate [5] per day respectively;

[3] Obtained from the Kessler Chemical Co., Philadelphia, Pa.

[4] Methyl linoleate was prepared from safflower oil in our laboratory. On the basis of iodine value determinations, the samples were shown to have a purity of 92 to 95%.

[5] Obtained from Biorganic Chemical Company, Los Angeles.

TABLE 1

Comparative effect of methyl linoleate, ethyl linolenate, and a mixture of methyl linoleate and ethyl linolenate on the survival of male rats which were subjected to 200 r/week (7 exposures) of x-irradiation

CATEGORY	GROUP NO.									
	1	2	3	4	5	6	7	8	9	10
Body weight at start of radiation, gm	181.7	198.2	191.4	201.2	200.2	196.1	195.1	198.0	190.0	199.0
Rats per group	13	14	13	14	14	14	14	14	13	14
Methyl linoleate given daily, mg	0	10	20	40	80	0	0	0	0	10
Ethyl linolenate given daily, mg	0	0	0	0	0	10	20	40	80	10
Average survival time, days[1]	40.2 ± 5.5	74.4 ± 8.4	75.2 ± 10.4	76.1 ± 10.0	82.2 ± 11.6	46.6 ± 8.8	64.9 ± 8.0	48.4 ± 5.4	43.6 ± 7.4	90.0 ± 8.2
M.D.:S.E.M.D.[2]		*3.4*	*3.1*	*3.2*	*3.5*	*0.6*	*2.6*	*1.0*	*0.3*	*5.1*
Average exposure, *r*	1046	1357	1276	1285	1214	1242	1300	1244	1057	1383
Mortality at 119 days, %	100	100	69.2	64.2	50.0	100	85.7	100	92.8	57.1

[1] Including the standard error of the mean calculated from formula $\sqrt{\Sigma D^2/n - 1}/\sqrt{n}$; "D" is the deviation from mean, "n" represents the number of observations.

[2] When the value exceeds 3, the results are considered to be highly significant.

group 10, 10 mg each of methyl linoleate and ethyl linolenate per day. The dosages were calculated on the basis of the free acid content. All doses were made up to 0.25 ml with ethyl laurate.

The rats were housed in individual cages during the experimental period in a room in which the temperature was thermostatically controlled. Food and water were given ad libitum. Body weights were recorded weekly. After a two-week orientation period following the initiation of supplementation, the rats were subjected to doses of x-irradiation of 200 *r* weekly for 7 weeks. The procedures for radiation were similar to those described earlier (Cheng et al., '52). The animals were observed for 10 weeks after the irradiation was concluded, during which period the experimental dietary regimen and supplementation were continued.

RESULTS AND DISCUSSION

Table 1 records the data on the comparative effect of methyl linoleate and of ethyl linolenate when given separately or in combination to protect male rats from x-irradiation injury when they were subjected to 7 doses of 200 *r* each, given at weekly intervals.

In groups 2 to 5, which received the methyl linoleate supplements at 4 levels, the average survival time is practically doubled from that of the control group (group 1) which received no essential fatty acids. The survival time in all of these groups is significantly greater than the control values in spite of the wide individual variations. However, there is not a proportional increase in average survival time with increasing dose of linoleate as has been reported earlier from this laboratory (Cheng et al., '54b). As a possible explanation for this variance from our previous results, it is possible that the age of the animals may account for the differences. In the earlier tests, the rats were 20 to 23 weeks old at the start of the tests while in the present case the experiments were initiated after 11 weeks.

In spite of the failure of the average survival time to exhibit a progressively increased value with increasing doses of linoleate, the degree of mortality at the termination of the experiment (119 days) does show a progressively lower value with the increased dosage of linoleate. While 100% of the animals had succumbed in the control group and in group 2 (10 mg linoleate), the percentage which had died in groups 3 to 5 was only 69.2, 64.2, and 50.0 respectively. This would indicate that the extent of mortality may be a better criterion with which to evaluate the protective effect of the essential fatty acids than is the average survival time.

In sharp contrast to the protective effect of linoleate, linolenate when given by itself was uniformly almost entirely ineffective at 10, 20, 40, or 80 mg daily. Some increase in survival time appeared to obtain in group 7 (20 mg linolenate) but this was not statistically significant. The average survival times for the rats receiving 40 and 80 mg of linolenate closely approximated that of the control group. The protective effect of linolenate is likewise unsatisfactory when based on the extent of mortality. Thus, in groups 6 to 9, which received 10, 20, 40, and 80 mg of linolenate respectively, the mortality at the termination of the test was 100, 85.7, 100, and 92.8% respectively.

When linolenate is fed together with linoleate, however, the protective action against x-ray is markedly increased; it would appear that linoleate produces a synergistic effect on the action of linolenate or vice versa. Thus, when 10 mg each of linoleate and linolenate were fed (group 10), the highest average survival time of any group was observed; it exceeded that not only of the groups receiving 10 and 20 mg of linoleate as supplement but even of group 5 which received 80 mg of linoleate daily. Moreover, the extent of mortality observed in group 10 was the lowest of any except group 5 (which received the highest dosage of linoleate).

The inactivity of linolenate in protecting against x-irradiation in the absence of linoleate is similar to the results obtained with these polyunsaturated acids on the growth of

rats receiving a fat-free diet as reported by Greenberg et al. ('50). Moreover, there was some augmentation in the effect on growth when these two acids were fed simultaneously although this effect was not as striking as the protection afforded by the combined acids noted here. The best explanation for this phenomenon would still seem to be that linoleate is needed to activate the linolenate.

If the effectiveness of a compound in protecting against the damage caused by x-irradiation is related to its ability to stimulate the growth of new tissue, then this would account for the ability of a compound to promote growth of rats on a fat-free diet. It would appear that linoleate is required to initiate the growth reaction of rats on fat-free diets both in the absence or presence of x-irradiation as a stress factor. When this basic requirement is met, linolenate then is able to potentiate the response as effectively or even more effectively than linoleate.

In a second series of tests on female rats, it was likewise observed that linolenate offered only slight protection against x-irradiation injury as contrasted with the marked potency exerted by equivalent amounts of linoleate. Although in the experiments the synergistic effect of linoleate on linolenate is not as clearly defined as in the tests with males, this phenomenon may be related to the marked variation in the requirement of essential fatty acids which obtains between the sexes.

In addition to the experiments reported here, a third series of tests was carried out with the same number of animals and with the same supplements as used in the first series; however, instead of the x-irradiation being given in 7 doses at weekly intervals, the rats were subjected to only two exposures of 400 *r* each at a 5-day interval. The results in this third series were somewhat less clear-cut than the first but did help to establish the ineffectiveness of linolenate and likewise the synergistic action of linoleate on linolenate.

SUMMARY

The ability of linoleic acid to protect animals against x-irradiation injury has again been confirmed. Linolenic acid exhibits only a slight and insignificant protective effect against x-irradiation in male rats as judged by average survival time and by the extent of mortality at the termination of the experiment. However, when linolenate is administered at a minimum level (10 mg) together with a minimum level of linoleate (10 mg), a marked increase in protective action against x-irradiation is obtained which suggests that a synergism exists. This behavior is similar to the action on growth of linolenate alone and with linoleate observed earlier in rats on fat-free diets. It is again suggested that linoleate is required to initiate the action of linolenate.

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NUTRITIONAL STATUS OF THE AGING [1]

V. VITAMIN A AND CAROTENE

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FOUR FIGURES

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Determination of serum vitamin A and carotene levels as well as 7-day diet records and physical examinations were obtained on 514 supposedly healthy men and women over 50 years of age, residents of San Mateo County, California. The methods and objects of the study have been described (Gillum and Morgan, '55). All participants were living in their own homes except 44 men over 60 years of age who were living in the county home. Blood analyses were made on only 30 of this last group.

The serum vitamin A and carotene levels were determined by the method of Bessey, Lowry, Brock and Lopez ('46).[2]

RESULTS

The results of these determinations are shown in table 1 and their distribution in figure 1. It is evident that there was no significant difference due to sex but an irregular decline

[1] This study was part of the Western Regional Research Project, W-4 on nutritional status of population groups. It was financed in part by funds appropriated under the Research and Marketing Act of 1946. Substantial help and cooperation were received from the Human Nutrition Research Branch, U. S. Department of Agriculture, the United States Public Health Service, the California State Department of Public Health and the San Mateo County Department of Public Health and Welfare.

[2] The serum analyses were done by John Barnwell and Esther Goossen.

in both levels with increasing age, particularly in the men. About one-third of all serum vitamin A levels were under 50 μg %, nearly two-thirds below 60 and about 95% below 80. Fifty-three per cent of all carotene levels were under 110

TABLE 1

Serum vitamin A and carotene levels of men and women over 50 years of age

AGE GROUP	NO. OF SUB-JECTS	SERUM VITAMIN A				SERUM CARQTENE			
		Median	Mean	Range	Stand-ard error	Median	Mean	Range	Stand-ard error
yrs.		*μg %*	*μg %*	*μg %*		*μg %*	*μg %*	*μg %*	
50–54									
Men	40	57	58	20–109	3	123	123	29–230	7
Women	46	54	57	17–131	3	108	118	46–307	8
55–59									
Men	38	62	61	31– 87	2	109	115	31–311	9
Women	48	56	57	29–120	3	110	129	48–429	10
60–64									
Men	34	56	54	9– 82	3	99	111	43–299	6
Women	52	58	56	20– 92	2	121	137	33–386	10
C.H. men [1]	7	42	49	33– 96	8	100	102	33–207	21
65–69									
Men	38	54	54	23– 88	2	109	130	47–311	11
Women	51	59	58	28– 87	2	101	117	25–269	8
C.H. men	10	46	49	30– 66	8	101	95	46–153	13
70–74									
Men	34	51	50	24– 94	3	110	113	43–217	8
Women	37	52	50	22– 77	3	104	112	49–246	7
C.H. men	8	43	46	32– 62	7	93	93	31–199	20
75–79									
Men	16	52	53	34–104	5	103	130	33–405	24
Women	25	52	50	15– 74	3	97	121	48–429	20
C.H. men	4	36	39	28– 54	6	80	89	78–121	11
80 +									
Men	15	50	47	18– 72	5	83	90	43–147	8
Women	10	53	53	46– 61	2	108	101	29–206	17
C.H. men	1		60				167		
Totals									
Men	215	56	56	9–109	3	108	118	29–405	9
Women	269	54	54	15–131	3	106	120	25–429	10
C.H. men	30	43	47	28– 96	8	95	98	31–199	16

[1] Men living in the county home.

μg % and more than 95% under 250. The distribution of the vitamin A levels was normal in both men and women but that of carotene was skewed so that 71% of all values fell between 45 and 150 μg % with only 4% below 45 and 25% above 150 (fig. 1).

Vitamin A and carotene intakes

The dietary content of vitamin A from both animal and plant sources was calculated in international units, expressed

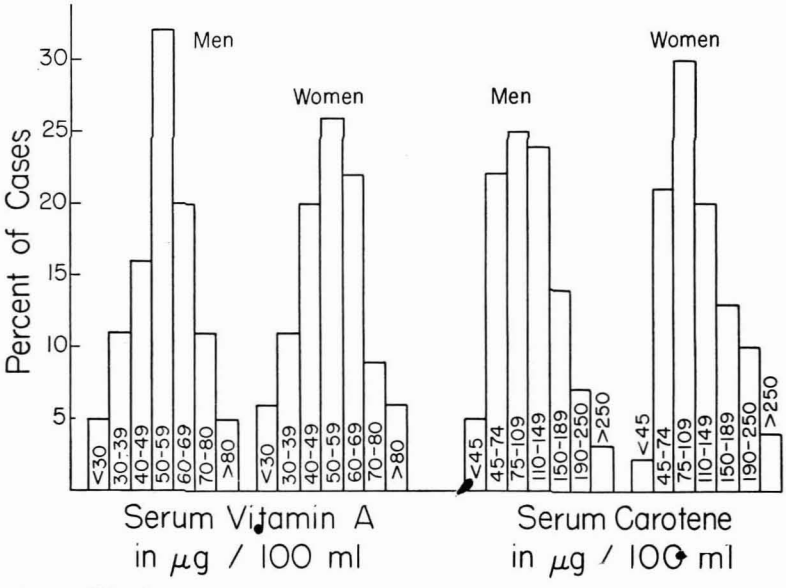

Fig. 1 Distribution of serum vitamin A and carotene levels in men and women over 50 years of age.

as a total and separated into preformed vitamin A and carotene (table 2). The international unit of preformed vitamin A was taken to be equivalent to 0.344 μg vitamin A, the value given the ester form, since nearly all food vitamin A is in that form. The carotenoid content of milk fat and egg yolk was ignored because of the wide seasonal changes in this constituent. Thus the vitamin A of these foods of animal origin

was considered all preformed vitamin A, leading to a slight overweighting.

The total vitamin A equivalent of the food eaten was calculated from the carotene and preformed vitamin A contents by assuming 3 I.U. of carotene to be equivalent biologically to 1 I.U. of preformed vitamin A. This was based on the conclusions of Hume ('51) from a study on human volunteers (Hume and Krebs, '49). The mean effective vitamin A daily intakes were then expressed as micrograms per kilogram of body weight (table 2).

It should be noted that this method of appraising the biological value of food carotenoids produces an underestimate since some of the provitamin A values were obtained by bioassay. Presumably these values are fully equivalent to those cited for vitamin A of animal origin. The compilation by Watt and Merrill ('50) was the chief source of the figures used in these calculations. These values were compiled from many sources and include the results of both chemical and biological analyses. Data making it possible to form a new table of vitamin A content of foods are needed in which the chemically determined values are reported uniformly in terms of micrograms of carotene or of vitamin A ester and the biologically determined values in international units or their equivalents.

The proportions of preformed vitamin A to carotene varied considerably. The women took 21 to 47% as carotene, mean 29, the men living in their own homes took 16 to 40% in that form, mean 27, and the men in the county home, 12 to 23%, mean 18. The total effective intake of vitamin A per kilogram of body weight per day varied in the 5-year age groups from 22 to 43 μg, mean 33, in the men living at home, from 19 to 38, mean 27, in the women, and from 15 to 27, mean 21, in the men in the county home. These figures are comparable with those obtained when the crude total intake in I.U. was used. Per kilogram per day by the latter calculation, the men living at home ingested 158, the women, 129, and the men in the county home 85 I.U. of vitamin A (table 2).

TABLE 2

Daily intakes of vitamin A and carotene

AGE GROUP	NO. OF SUB-JECTS	BODY WT.	MEAN DIETARY INTAKES					VIT. A EQUIVALENTS [1] FROM THE DIETS					DIETARY + SUPPLEMENTS VITAMIN A
			Carotene	Preformed vit. A	Total	S.E.[2]	Per kg	From carotene	From preformed vit. A	Total	From carotene	Per kg	Total I.U. × 1000 Mean
yrs.		*kg*	*I.U. × 1000*	*I.U. × 1000*	*I.U. × 1000*		*I.U.*	*μg*	*μg*	*μg*	*%*	*μg*	
50–54													
Men	37	74.1	5.41	4.27	9.68	1.11	132	620	1468	2088	30	28	10.23
Women	40	68.2	4.63	2.22	6.85	0.54	100	531	764	1295	41	19	8.36
55–59													
Men	36	74.5	6.29	6.63	12.92	1.83	173	721	2281	3002	24	40	13.49
Women	48	65.9	5.02	3.93	8.95	1.12	136	576	1352	1928	30	29	10.45
60–64													
Men	34	74.5	6.07	6.84	12.91	2.67	173	696	2353	3049	23	41	13.42
Women	32	65.4	4.57	5.48	10.05	1.62	153	524	1985	2509	21	38	11.31
C.H. men [3]	11	66.4	2.53	2.92	5.45	0.56	82	290	949	1239	23	19	
65–69													
Men	38	70.9	4.50	3.16	7.66	0.77	108	516	1087	1603	32	22	9.14
Women	51	65.0	4.99	3.92	8.91	1.91	137	572	1348	1920	30	29	11.31
C.H. men	16	61.8	2.05	3.41	5.46	0.62	88	235	1174	1409	17	23	
70–74													
Men	34	70.9	5.13	4.13	9.26	1.04	130	588	1420	2008	29	28	10.04
Women	37	63.6	4.40	3.03	8.43	1.24	132	505	1386	1891	26	30	11.07
C.H. men	8	67.7	2.31	3.48	5.79	0.70	85	265	1196	1461	18	21	
75–79													
Men	16	70.5	8.19	4.06	12.25	3.73	193	939	1397	2336	40	33	13.19
Women	25	63.2	5.15	1.90	7.05	1.17	111	592	653	1246	47	20	8.45
C.H. men	5	60.0	1.78	4.17	5.95	1.21	97	204	1450	1654	12	27	
80 +													
Men	15	66.8	3.87	7.01	10.88	3.46	163	444	2408	2852	16	43	12.55
Women	10	55.9	3.19	2.09	5.28	1.31	95	366	719	1085	33	19	6.28
C.H. men	4	64.5	1.96	2.16	4.12	0.82	64	225	748	973	23	15	
Totals													
Men	210	72.3	5.57	5.07	10.64	1.78	158	640	1747	2387	27	33	11.47
Women	263	65.4	4.64	3.81	8.45	1.31	129	532	1301	1833	29	27	10.20
C.H. men	44	64.0	2.18	3.27	5.45	0.71	85	240	1114	1354	18	21	5.45

[1] Three international units carotene ≙ 1 I.U. vitamin A ≙ 0.344 μg vitamin A ester.
[2] Standard error.
[3] Men living in the county home.

Twenty-eight of the men and 55 of the women took vitamin A supplements either in multivitamin preparations or separately more or less regularly. The mean daily dose was 6,470 I.U. for the men and 8,368 for the women. In addition, 13 men and 14 women took vitamin supplements but the amounts and kind were not known Those who took vitamin A supplements were well distributed in all age groups. The average total intake of all vitamin A including supplements is estimated for the 5-year age groups in table 2.

Relationship of intakes to serum levels

The total vitamin A intakes of all the subjects were divided into three groups including respectively 36, 26, and 44% of the women and 23, 26, and 51% of the men. These groups had daily intakes of less than 5,000 I.U. total vitamin A, 5,000 to 8,000, and over 8,000, including vitamin supplements. The vitamin A serum levels were divided also into three groups, less than 50 μg %, 50 to 65, and over 65, since these values also represented a fairly even division of the whole series. The percentage of men and women having each of the three ranges of vitamin A intakes within each of the three groupings of serum vitamin A levels is shown in figure 2. There is an obvious increase in the percentage of those taking the largest amount of the vitamin as the serum levels increase. The opposite is true for those having the lowest vitamin intakes

When a similar comparison was made between intakes and serum levels of carotene a higher correlation, $r^z = +0.37$, was found. The intake groupings in this case were less than 1.8 mg of carotene, 1.8 to 3.6 and more than 3.6. In this case each I.U. of plant origin was given the value of 0.6 μg of carotene. The serum level ranges were less than 75, 75 to 150 and over 150. These again represented nearly equal divisions of all the records. The striking correlation of frequency of low intakes in those having low serum levels and of high intakes in those with high serum levels is shown in figure 3. This is to be expected since serum carotenes reflect intake only.

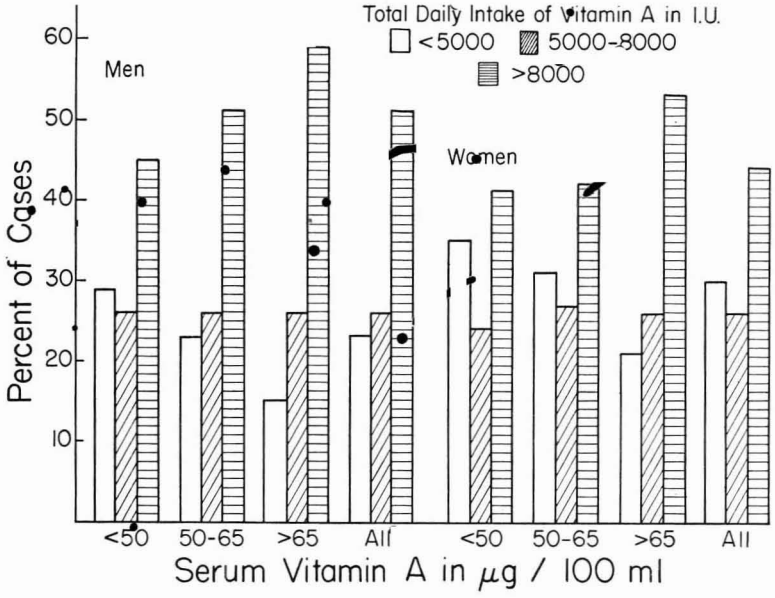

Fig. 2 Relation of total vitamin A intake to serum vitamin A levels.

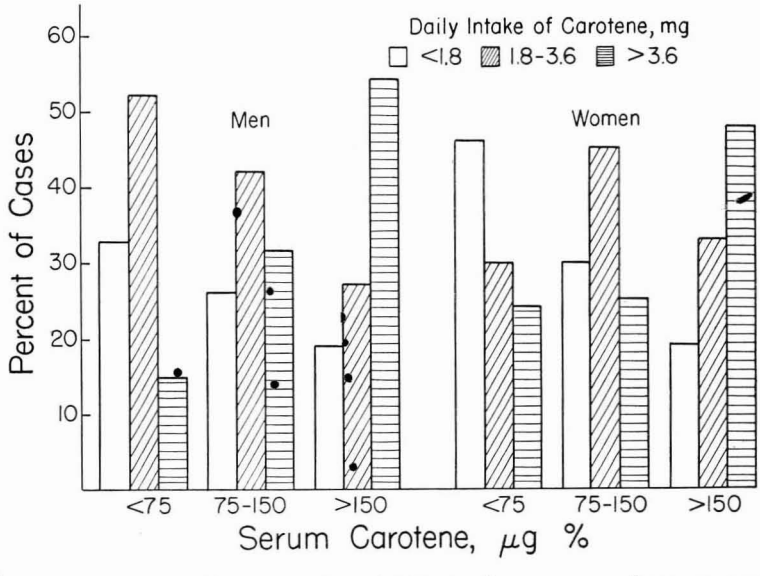

Fig. 3 Relation of dietary carotene intake to three ranges of serum carotene levels.

When the serum carotene values are plotted against the mean total carotene intakes the positive correlation appears clearly (fig. 4). When the carotene intakes were expressed as milligrams per kilogram of body weight practically the same curves were obtained.

A comparison of total vitamin A intake with the serum carotene levels yielded a less significant relationship. The mean dietary intake of men with serum carotene under 75

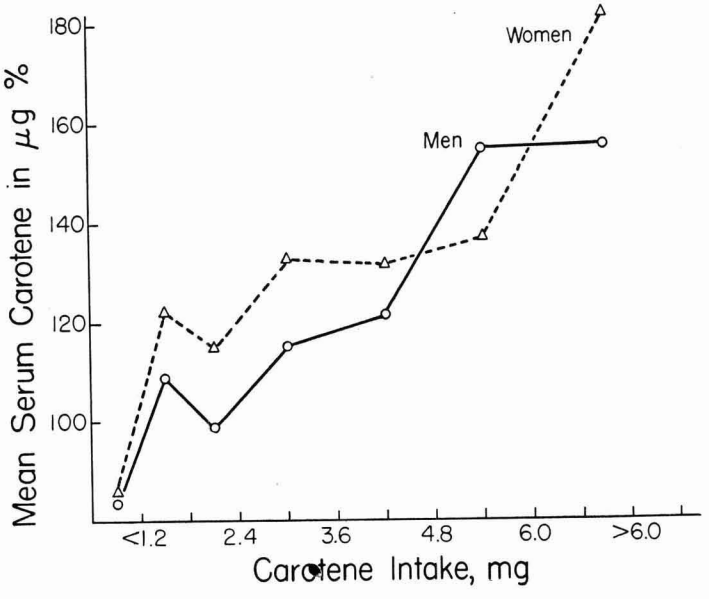

Fig. 4 Correlation of mean serum carotene levels with corresponding carotene intakes.

μg % was 12,018 I.U., 75 to 150 was 9,720, and over 150, 14,685. The corresponding figures for the women were 7,000, 9,000 and 14,250. Such relationship as appears may probably be ascribed to the carotene fraction of the intake. Merrow et al. ('52) reported similar correlations.

Physical signs of possible vitamin A deficiency

In table 3 are listed the frequencies of occurrence of changes in eyes and skin which have been ascribed to vitamin A defi-

ciency.[3] None of these signs occurred in a significant number of the subjects, except for thickening of the bulbar conjunctiva. This latter sign was so frequently noted, in 94.1% of all subjects, that its significance as a true vitamin A deficiency symptom rather than as a normal effect of aging, must be questioned. There was no difference in serum vitamin A levels between those who presented this sign and those who did not, nor was there any relationship to age or sex in its occurrence. The same may be said of the appearance

TABLE 3

Physical signs of possible vitamin A deficiency in 514 men and women over 50 years of age

SYMPTOMS	PER CENT OF SUBJECTS			
	Degree			
	Mild	Moderate	Severe	TOTAL
Bulbar conjunctiva				
Thickening	54.8	38.5	0.8	94.1
Increased vascularization	3.9	0.2	..	4.1
Palpebral conjunctiva				
Folliculosis	2.5	0.2	..	2.7
Spots	9.6 (1 spot)	1.6 (2 spots)	..	11.2
Skin xerosis	21.2	6.4	0.2	27.8
Skin folliculosis	0.8	..	0.2	1.0
Hyperkeratosis	7.4	2.0	..	9.4
Follicular plugs	2.7	0.2	..	2.9

of Bitot's spots, and the other changes in the conjunctiva as well as in the skin. For example, the mean serum vitamin A level of persons without thickening of the bulbar conjunctiva was 53 μg %, of those showing this sign, 55. Those with and without skin xerosis had the same mean levels, as did those with and without hyperkeratosis. In both xerosis and hyperkeratosis however there was a definite increase in number of subjects showing this sign with advancing age. Xerosis was more frequently seen in the women, hyperkeratosis in the men.

[3] We are indebted to Sheldon Dray, U. S. Public Health Service for the assessment of these signs.

The dietary and supplementary vitamin A and carotene intakes of the persons who showed these physical signs were widely varied, and showed no correlation with incidence of the signs.

Effect of mineral oil intake

Ten of the subjects reported intake of mineral oil three to 7 times per week. Six of these who took the oil every day had average serum vitamin A and carotene levels of 46 and 108 μg % respectively and the 4 who took it three or 4 times a week, 72 and 103. The total daily vitamin A intake of this group was 8,240 I.U. of which 6,000 I.U. were from plant sources. The average value of serum carotene of all subjects with this intake was about 125 (fig. 4). One 82-year-old man who took mineral oil daily had a serum vitamin A value of 30 μg % and carotene 43, although his intake of total vitamin A was reported as 5,292, 82% of which was from carotene. Two others in this group had serum vitamin A levels of 33 and 34 but carotene levels of 122 and 224. The intakes of these subjects were 8,280 and 9,791 I.U. total vitamin A daily but 91 and 85% of these were from plant sources. A 75-year-old man in this group who took supplements so that he had intakes of 69,350 I.U. of vitamin A and 17,288 of carotene had a serum vitamin A level of only 54 and serum carotene level of 69. Alexander et al. ('47) found a decrease of 50% in plasma carotene levels with prolonged daily ingestion of mineral oil, but Steigman et al. ('50) reported large differences in the effect of the oil due to variations in amount and time of dosing.

DISCUSSION

The tendency toward a decrease in serum vitamin A level with aging has been seen previously by Rafsky and Newman ('48). In subjects past 65 years of age 65% had serum carotene below 100 μg % and 82% had serum vitamin A below 40 μg %. This may be compared with about 45% and 17% below these levels in the present study. Most of the studies, how-

ever, indicate little if any effect of age upon these blood levels. Haig and Patek ('42) and Campbell and Tonks ('49) found no differences in their series of young and old adults. Kirk and Chieffi ('48), in a study of 155 institutionalized middle-aged and elderly subjects, saw no significant difference between the means of their plasma vitamin A and carotene levels and those of a group of subjects 16 to 39 years of age. Yiengst and Shock ('49) who examined 126 similar elderly males also saw no changes in these levels with age. It must be noted that in all of these studies the subjects were living in institutions and were possibly survivors of more or less stressfull lives. These observations may be compared with the records of the men in the county home in this study rather than with those of the men living in their own homes. The intake of preformed vitamin A equivalent of the former was 21 μg per kilogram, only 62% of that of the men living at home. Their circulating vitamin A and carotene were likewise low, 84 and 83% of those found in the men living in their own homes.

The trend toward a lowering of these levels with age shown in our records is not striking except as it is seen in relation to the generous intakes of vitamin A maintained by the majority of the older subjects. The men 50 to 59 years old had a mean serum vitamin A of 60 μg %, 60 to 69 years old 54, and over 70, 50. The women 50 to 59 and 60 to 69 years old had levels of 57 μg % and those over 70, of 50. The differences between the mean values in the 50- to 60-year-old groups and those of the group over 70 are significant in both men and women. The dietary intakes of preformed vitamin A equivalents per kilogram body weight for these age groups were for the men, 34, 31, and 33 μg, for the women 24, 32, and 25. The corresponding serum carotene levels by decades were 119, 116, and 109 μg % in the men and 123, 127, and 113 in the women. The average daily carotene intakes in international units per kilogram body weight of these groups of men were each 78 and of the women 72, 74, and 72.

The mean levels of serum vitamin A found in this study were higher than those usually reported. The values of 56 and 54 μg % for men and women respectively may be compared with the 35 (117 I.U.) quoted by Moore and Leitner ('49) as the average for 325 men and women reported in 8 American and British studies. Moore and Leitner found 36 (121 I.U.) as the average of their own determinations on 195 adults, about one-half of whom were nospital patients. About 64% of these values were in the range of 24 to 48 μg %.

The mean serum carotene levels, 118 and 120 μg % for men and women respectively were closer to those usually reported. Moore and Leitner ('49) quoted 177 (295 I.U.) from the 8 studies previously mentioned and 92 (153 I.U.) from their own.

Reports of sex differences in blood vitamin A and carotene levels generally show a trend toward higher vitamin A levels in males and higher carotene levels in females (Moore and Sharman, '51; Hoffman et al., '50; Rafsky et al., '47). In two studies, those of Haig and Patek ('42) and Abels et al., ('41), the carotene level was found to be higher in the males than in the females. It has been suggested that carotene intakes per kilogram of body weight may often be higher in females and that their tolerance for carotene may therefore be more frequently exceeded. This was not true in this study, however, since the mean daily intakes of carotene per kilogram of body weight of men and of women were 77 $\pm$ 6 and 71 $\pm$ 8 I. U. respectively, with median serum carotene levels of 108 and 106. The median vitamin A and carotene serum levels of the 5-year age groups (table 1) showed no consistent significant differences between the sexes. Kirk and Chieffi ('48) also found no sex differences in these blood constituents.

The comparison of intakes with circulating levels of vitamin A and carotene is complicated by the rapid storage of excess vitamin A and the somewhat less rapid transformation of ingested carotene into vitamin A for both circulation and storage. Large increases in carotene intake may raise the carotene level in man remarkably and continuously without

affecting the serum vitamin A value (Hoch, '43). Similar increases in vitamin A intake affect the serum levels of vitamin A within a few hours but within 24 hours the resting level is again restored. Meanwhile the carotene level may not be affected. Thus, dietary carotene directly affects the serum carotene level but only indirectly the circulating vitamin A.

In this study a direct correlation of intake of carotene with its serum levels was convincingly demonstrated (figs. 3, 4). The carotene intakes were not similarly related to the serum vitamin A levels nor were the total vitamin A intakes so closely related to the carotene levels. The latter value appears to be indicative of the recent carotene intake only and not to be directly significant with regard to the vitamin A status. Its routine determination therefore may be of value only as a check on dietary records and on the efficiency of absorption and transformation of food carotenoids.

The frequently reported lack of correlation of certain conjunctival and skin changes with blood vitamin A levels (Youmans et al., '44; Anderson and Milam, '45) was confirmed again in this study. Darby and Milam ('45) found conjunctival changes in 75% of adults examined but no greater incidence in those with low plasma vitamin A levels or low dietary intake than in the others. Chieffi and Kirk ('49), however, reported higher incidence of hyperkeratosis and conjunctival thickening in middle-aged and old subjects with low plasma vitamin A levels (1 to 15 μg %) than in those with higher concentrations (25 to 60 μg %). In aging subjects the generally quoted signs of low vitamin status in eyes and skin appear to be more often seen than in young people but their dependence upon actual vitamin A deficiency is questionable.

SUMMARY

Serum vitamin A and carotene determinations, physical examinations and 7-day dietary records were obtained on 514 supposedly healthy men and women over 50 years of age. Thirty of the men were living in the county home, the others in their own homes.

A small decline in both vitamin A and carotene serum levels occurred with age in both men and women, the mean values by decades being, in micrograms per cent, 60, 54 and 50 for the men and 57, 57, and 50 for the women. The corresponding carotene values were 119 116, 109 for the men and 123, 127, 113 for the women. No significant sex differences were found.

Only 5.6% of the subjects had serum vitamin A levels below 30 μg % and 5.2% above 80. Nearly half of both men and women had serum vitamin A levels between 40 and 60 μg %. The serum carotene levels varied more widely, with about 62% falling between 60 and 150 μg %.

The dietary intakes were calculated for preformed vitamin A and for carotene separately and the probable vitamin A equivalent determined. This was found to be 2,387 μg per day for the men living in their own homes, 1,883 for the women and 1,354 for the men in the county home. This amounted to 33, 27, and 21 μg per kilogram of body weight. Twenty-seven per cent of the intake of the men living in their own homes was from carotene, 29% of that of the women and 18% of that of the men in the county home. About 11% of the men and 20% of the women took vitamin A supplements more or less regularly. When these were included the average total intakes were 11,470 and 10,200 I.U. daily for the men and women living in their own homes as compared with 10,640 and 8,450 when food sources only were used.

A slight positive correlation between total vitamin A intake and serum vitamin A level was established for both men and women. The correlation between serum carotene and carotene intake was more pronounced, $r^z = 0.37$.

Thickening of the bulbar conjunctiva was noted in 94.1% of the subjects but this was not more marked in those with low than in those with high serum levels of vitamin A. The same was true of the other changes in the conjunctiva and skin. There was more relationship to age than to vitamin A levels or intakes in the distribution of these signs.

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NUTRITIONAL STATUS OF THE AGING [1]

VI. SERUM PROTEIN, BLOOD NONPROTEIN NITROGEN, URIC ACID AND CREATININE

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SIX FIGURES

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The nonprotein nitrogenous constituents of the blood were studied in 255 and serum protein levels in 573 men and women over 50 years of age, all living in their own homes in San Mateo County, California, except for 44 men who were living in the county home. Physical examinations and 7-day dietary records were also obtained in all cases. The methods and objectives of this study have been described (Gillum and Morgan, '55).

PROCEDURE

The blood used for the protein-free filtrates was drawn by venipuncture and a portion placed at once in a flask containing a small amount of lithium oxalate. The flasks were shaken and the blood transferred to tubes which were at once frozen and kept frozen until they were analyzed. Portions of blood were also transferred to unoxalated tubes which were centrifuged after standing 15 to 20 minutes, the serum

[1] This study was part of the Western Regional Research Project, W-4 on nutritional status of population groups. It was financed in part by funds appropriated under the Research and Marketing Act of 1946. Substantial help and cooperation were received from the Human Nutrition Research Branch of the United States Department of Agriculture, the United States Public Health Service, the California State Department of Public Health and the San Mateo County Department of Public Health and Welfare.

separated and the total serum proteins determined immediately [2] by the specific gravity method of Phillips et al. ('43).

The protein-free blood filtrates were prepared according to the method of Folin and Wu ('19). The same filtrate was used for nonprotein nitrogen, uric acid and creatinine.

Nonprotein nitrogen was determined by micro Kjeldahl process using steam distillation for recovery of the ammonia in boric acid and back titration with standard acid. Satisfactory blanks and recoveries from ammonium sulfate and urea were established. [3]

The method of Folin and Wu ('19) was used for creatinine.

The method chosen for uric acid after much consideration was that of Brown ('45). Because of lack of specificity of the methods used for blood uric acid analysis all uric acid data reported must be considered in the light of the procedures used. The Folin ('22) method long in use may not yield the full uric acid values.

The methods which include the use of the enzyme uricase may yield either low or accurate values while the ferricyanide methods may include nonuric acid-reducing substances. Added uric acid has been recovered poorly in nearly all the methods mentioned. Brown ('45) proposed the use of modified amounts of urea and sodium cyanide solutions to overcome the color-inhibiting effect of certain substances present in the tungstic acid blood filtrates. A modified Folin-Denis tungstate-phosphate uric acid reagent was also used and comparisons made with the color produced in pure uric acid solutions. When the uricase enzyme treatment was used on parallel samples the reduction in apparent uric acid content was small, on the order of 10%. The range of concentration in human blood samples by the method of Brown is 2 to 3.5 mg %, which compares well with that obtained by the Folin ('33) isolation procedure. This method was therefore adopted as suitable for routine use and likely to yield true blood uric acid values.

[2] The serum protein determinations were made by Mildred Snowden.

[3] The nonprotein nitrogen analyses were made by John F. Taugher.

Centrifuged urine samples were tested qualitatively for albumin by the addition of an equal volume of 3% sulphosalicylic acid solution. The degree of cloudiness or appearance of precipitate on standing was estimated as indicating a trace or 1+ to 4+ albuminuria.

TABLE 1

Serum protein and nonprotein nitrogen of men and women over 50 years of age

AGE GROUP	NO.	SERUM PROTEIN			NO.	NONPROTEIN NITROGEN		
		Mean	Range	Stand. error		Mean	Range	Stand. error
		gm %	*gm %*			*mg %*	*mg %*	
50–54								
Men	45	6.5	5.8–7.2	0.05	24	37	27–48	1.2
Women	49	6.5	5.6–7.2	0.05	19	34	15–42	1.4
55–59								
Men	41	6.5	6.2–7.2	0.04	21	38	24–51	1.7
Women	54	6.5	5.8–7.2	0.04	25	33	24–42	1.0
60–64								
Men	36	6.5	5.8–7.2	0.06	12	39	32–47	1.3
Women	57	6.4	5.2–7.7	0.05	26	33	21–47	1.3
C.H. Men [1]	11	6.7	5.8–7.4	0.16				
65–69								
Men	39	6.4	5.6–6.9	0.06	21	38	28–45	1.0
Women	57	6.5	5.8–7.7	0.04	28	34	17–49	1.1
C.H. Men	16	6.6	5.8–7.0	0.08				
70–74								
Men	37	6.5	5.8–6.9	0.05	16	35	26–46	1.3
Women	40	6.4	5.8–6.9	0.05	24	36	26–44	0.8
C.H. Men	8	6.6	6.2–7.0	0.10				
75–79								
Men	19	6.4	5.8–7.2	0.08	12	36	24–43	1.8
Women	25	6.4	5.8–6.9	0.08	11	35	28–46	1.9
C.H. Men	5	6.4	5.8–7.0	0.19				
80 +								
Men	18	6.5	6.2–7.2	0.08	11	35	22–42	1.7
Women	12	6.4	5.8–6.9	0.11	5	33	30–38	1.7
C.H. Men	4	6.6	6.5–6.8	0.07				
Totals								
Men	235	6.47	5.6–7.2	0.06	117	38	22–51	1.4
Women	294	6.44	5.2–7.7	0.05	138	34	15–49	1.2
C.H. Men	44	6.60	5.8–7.4	0.12				

[1] Men living in county home.

RESULTS AND DISCUSSION

Total serum protein

There was only one person, a woman, with serum protein level below 5.5% and only 5 men and 4 women with levels above 7.0 gm%. Only 6 to 7% of the values were below 6.0% Thirty-four per cent of the women and 38% of the men had serum protein levels between 6.0 and 6.5, and 60% of the women and 54% of the men between 6.5 and 7.0 (table 1).

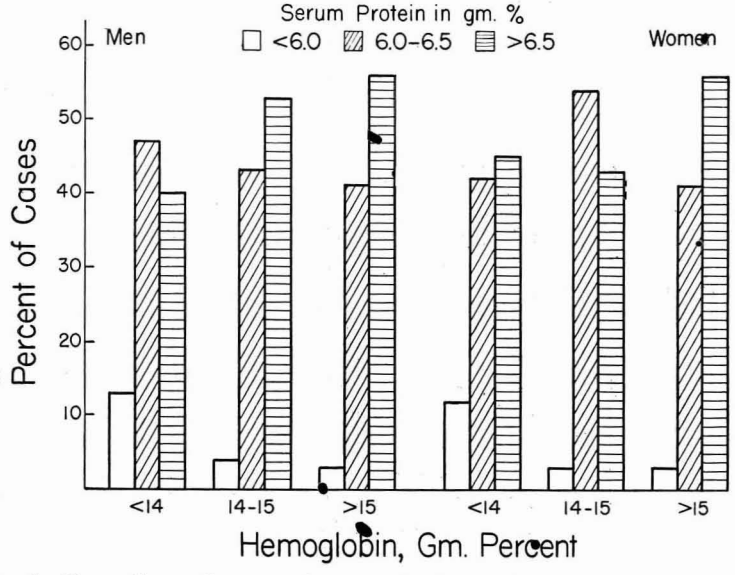

Fig. 1 Proportions of men and women having certain ranges of total serum protein among those with three groups of hemoglobin levels.

No correlation with dietary protein could be detected nor was there any regular change with age or difference between the sexes. When the hemoglobin levels were compared with these serum protein levels, however, a small positive correlation was detected (fig. 1). Since a moderate positive correlation had been found between hemoglobin levels and protein intakes (Gillum and Morgan, '55) it is likely that the serum protein values were at least remotely affected by the

diet. It is clear, however, that in this group the hemoglobin level was a better index of protein nutritional status than was the total serum protein.

The mean serum protein level of the 235 men living in their own homes was 6.47 ± 0.06 gm% and of the women 6.44 ± 0.05. The 44 men living in the county home had a mean level of 6.60 ± 0.12 gm%. These values are lower than those usually quoted for normal young adults. Albritton ('52) gives 7.13 or 7.2 as the normal mean, with a range of 6.5 to 7.9 gm %; Krebs ('50) suggests 6.72; Bruckman et al. ('30), using a limited number of healthy subjects, found 6.5 to 8.4 to be the limits of serum protein concentration. The average values were 6.9 gm % for males and 7.6 for females.

Olbrich ('48) also found lower levels of plasma protein in 78 old subjects, 5.70 gm %, with a range of 4.02 to 7.14. These people were living in an institution which provided adequate dietary protein. Rafsky et al. ('52) did repeated studies on the blood of 31 similar persons 65 to 95 years old and found the average serum protein level to be 6.97 ± 0.30, with a range of 6.12 to 7.88 gm %. The albumin fraction, however, was found to be significantly decreased and the beta globulin increased, with lowered albumin-globulin ratio, as compared with similar values in normal young adults. This latter finding is repeated in nearly every report of fractionation of serum proteins in old people (Bock, '48).

No correlation of serum protein concentration with dietary protein was seen by Youmans et al. ('43) in a survey of some 1200 persons, nearly half of whom were over 21 years of age. The mean in the adults was 6.86 for the men and 6.94 gm % for the women. In spite of a relatively low protein intake there were few cases of low total serum protein and even of hypoalbuminemia. Edema was seen in 3% but this was not associated with low serum proteins nor with low protein intakes.

There was only one case of edema in the present series and this was not due to disturbance of the protein nutrition.

The failure of correlation of serum protein levels with protein intakes was striking in this study (table 2). The average protein intakes were adequate, 1.1 gm per kilogram for the men living at home and 0.9 for the women and other men in the county home. Only 12 men had a daily intake of less than 60 gm of protein and only 32 women had less than 50 gm. The calories were also sufficient in practically all cases (Gillum and Morgan '55). Nevertheless the men and women living at home had a rather narrow range and low mean serum protein

TABLE 2

Serum protein levels compared with daily protein intakes

PROTEIN INTAKES	TOTAL SERUM PROTEIN, gm %				ALL LEVELS NO. OF SUBJECTS
	< 6.0	6.0–6.5	6.5–7.0	> 7.0	
gm	%	%	%	%	
Men					
< 60	5	45	40	10	20
60–79	4	39	56	1	72
80–90	13	43	43	0	39
> 90	8	32	57	3	80
					211
Women					
< 50	3	35	62	0	68
50–59	11	29	59	1	70
60–70	9	36	52	3	57
> 70	3	38	58	1	74
					269

levels. The men in the county home had a relatively higher level in spite of their lower intake of protein. The hemoglobin levels of these men were also equal to those of the men living in their own homes.

Nonprotein nitrogen

There appeared to be a higher average concentration of nonprotein nitrogen in the serum of the men than in that of the women up to 70 years of age. The mean for the 117 men was 38 ± 1.4 mg%; for the 138 women 34 ± 1.2. After 70 years

of age there was some decline in the level in the men but not in the women (table 1). The distribution is shown in figure 2.

The normal range for blood nonprotein nitrogen is usually given as 18 to 40 mg %, with a mean of 25 (Berglund, '22). Hammett ('20), however, found 35.6 mg % as the mean of 60 analyses of blood of 9 normal young adults. Cramer and Winnick ('43) found 25.7 the mean for normal adults. Aaltonen ('39) reported that the nonprotein nitrogen in 24 of

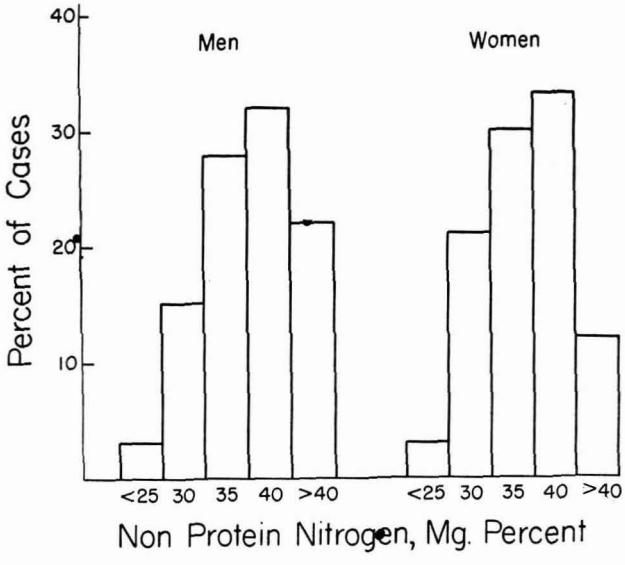

Fig. 2 The distribution of blood nonprotein nitrogen levels.

47 persons 80 to 93 years old was more than 45 mg %. Pathology may not have been ruled out in all these cases. Renal clearance, which is variable, especially in old people, affects all fractions of the serum nonprotein nitrogen and complicates the interpretation of these data.

Some direct correlation of protein intake with nonprotein nitrogenous levels was found in the men in this study but none, or a negative one, in the women. In figure 3 are shown the proportions of men having less than 35, 35 to 40 and more than 40 mg % of nonprotein nitrogen among those who ingested

less than 70, 70 to 90 and more than 90 gm of protein per day. A similar division of the women into those with less than 30, 30 to 35 and more than 35 mg % of nonprotein nitrogen was made and these were sorted for increasing amounts of protein intake. A direct but not striking relationship between blood nonprotein nitrogen and the level of dietary protein is seen in the comparison for the men but none for the women. Kountz

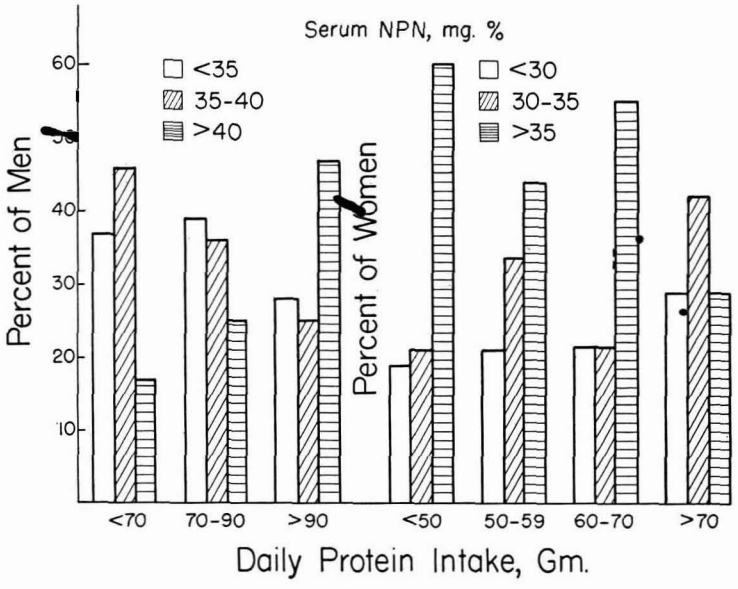

Fig. 3 The correlation of nonprotein nitrogen blood levels with dietary protein in men and lack of correlation in women.

et al. ('53) have also noted this relationship and suggest that excessive protein intake by old people may involve some renal difficulty.

Uric acid

Since the choice of analytical method for blood uric acid affects the relative magnitude of the values obtained, blood samples from 9 men and 14 women 18 to 40 years of age were analyzed by the method adopted for comparison with the samples from the older subjects. The mean for these 23 young-

er people was 2.53 mg % for the men and 2.33 for the women with a total range of 2.0 to 3.1 (table 3). The mean for the 116 older men was 3.24 and for the 137 older women 2.98 with a range of 1.4 to 5.5 mg %. The men, in nearly all groups up to 70 years of age, had higher blood uric acid levels than the

TABLE 3

Uric acid, creatinine and uric acid: creatinine ratios in serum of men and women 18 to 40 and over 50 years of age

AGE GROUP	NO.	URIC ACID			NO.	CREATININE			URIC ACID: CREATININE
		Mean	Range	Stand. error		Mean	Range	Stand. error	
yrs.		*mg %*	*mg %*			*mg %*	*mg %*		
18–40									
Men	9	2.53	2.2–3.1	0.13	9	1.27	1.1–1.6	0.10	1.99
Women	14	2.33	2.0–2.8	0.08	14	1.18	1.0–1.8	0.07	1.97
50–54									
Men	23	3.33	2.1–5.5	0.18	20	1.51	1.0–2.4	0.08	2.20
Women	19	2.92	1.6–5.2	0.21	17	1.38	1.0–2.0	0.07	2.11
55–59									
Men	21	3.29	1.5–4.4	0.16	18	1.50	1.1–1.9	0.08	2.19
Women	25	3.03	1.4–5.1	0.19	21	1.55	1.1–2.6	0.10	1.95
60–64									
Men	12	3.65	2.8–5.2	0.25	12	1.68	1.2–2.8	0.14	2.18
Women	25	3.03	1.7–4.4	0.15	22	1.51	1.1–2.2	0.07	2.00
65–69									
Men	21	3.25	1.8–4.4	0.17	16	1.58	1.1–2.5	0.11	2.05
Women	28	3.06	1.6–5.5	0.17	27	1.47	0.6–2.3	0.08	2.08
70–74									
Men	15	3.17	1.9–4.9	0.24	14	1.41	0.7–2.2	0.12	2.25
Women	24	3.16	1.6–4.5	0.16	23	1.49	0.8–2.6	0.10	2.12
75–79									
Men	13	2.86	1.4–4.1	0.19	9	1.41	0.8–1.8	0.12	2.03
Women	11	2.48	1.6–3.8	0.19	11	1.67	1.3–2.2	0.09	1.48
80 +									
Men	11	2.93	2.0–3.9	0.18	9	1.60	1.3–2.2	0.13	1.83
Women	5	2.40	1.7–2.9	0.21	5	1.42	1.0–2.7	0.09	1.69
All, 50–80 +									
Men	116	3.24	1.4–5.5	0.19	98	1.53	0.7–2.8	0.11	2.12 ± 0.02
Women	137	2.98	1.4–5.5	0.18	126	1.50	0.6–2.7	0.08	1.99 ± 0.02

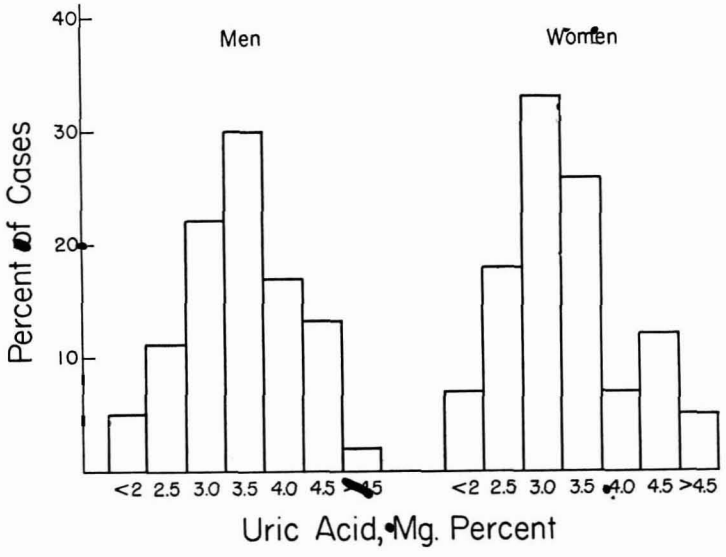

Fig. 4 The distribution of blood uric acid levels.

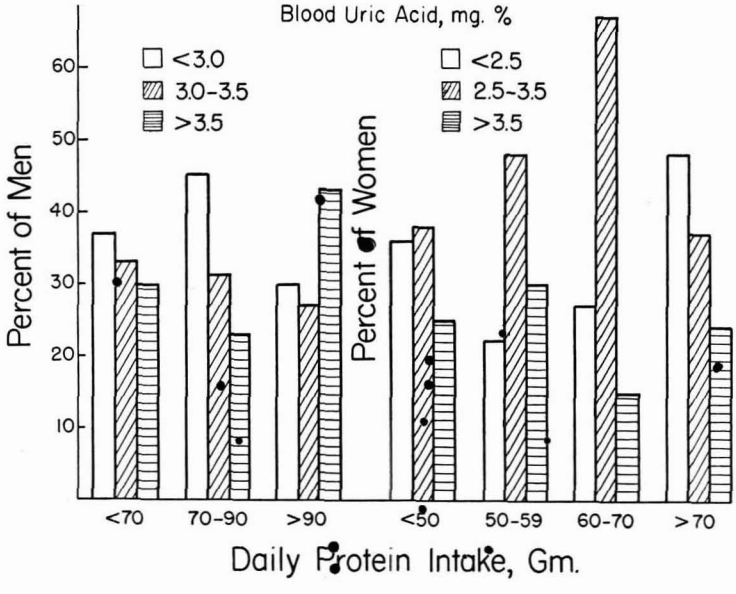

Fig. 5 The correlation of blood uric acid levels with dietary protein in men, but not in women.

women although the differences were not always significant. After 75 years of age the levels in both men and women decreased. The distribution of the uric acid levels is shown in figure 4.

Correlations of these levels with protein, fat and ascorbic acid intakes were attempted but none were positive except that between dietary protein and blood uric acid levels in the men (fig. 5).

The level of blood uric acid has been shown to rise markedly with extremely large fat intakes (Harding et al., '25) possibly due to ketosis. This is accompanied by decrease in uric acid excretion. Increase in protein intake at least temporarily increases uric acid output with decrease in circulating level (Folin et al., '24). The declining renal clearance known to occur in old age (Shock, '52) may account for the failure of customary high fat intake to produce any marked effect on the serum uric acid level or of high protein intake to reduce that level. The positive correlation between uric acid levels and protein intakes in the men may well be due also to the deterioration of the kidney circulation.

It has been reported previously (Praetorius, '51) that men had higher plasma uric acid levels than women but that aging exerted no consistent effect. Gertler and Oppenheimer ('53) confirmed the difference due to sex. In both of these studies the method used for uric acid analysis yielded consistently higher values than that used in this study and the differences in mean serum uric acid levels due to sex were also greater.

Creatinine

The blood creatinine levels in the older men and women were higher than in the young adults. No difference due to either sex or age could be discerned among the older subjects but the 23 young adults had significantly lower levels (table 3). The distribution is shown in figure 6. The range for blood creatinine quoted by Folin and Svedberg ('30) was 1.07 to 1.45, mean 1.18 mg %, in 19 young adults. Hammett ('20) found 1.3 mg % as the mean in 60 tests on blood filtrates of 9

young adults. The true creatinine values in plasma or serum are generally lower than in whole blood filtrates such as were used in this study because of the presence of non-creatinine chromogens in the cells.

No correlation of creatinine with protein intake was seen in any group.

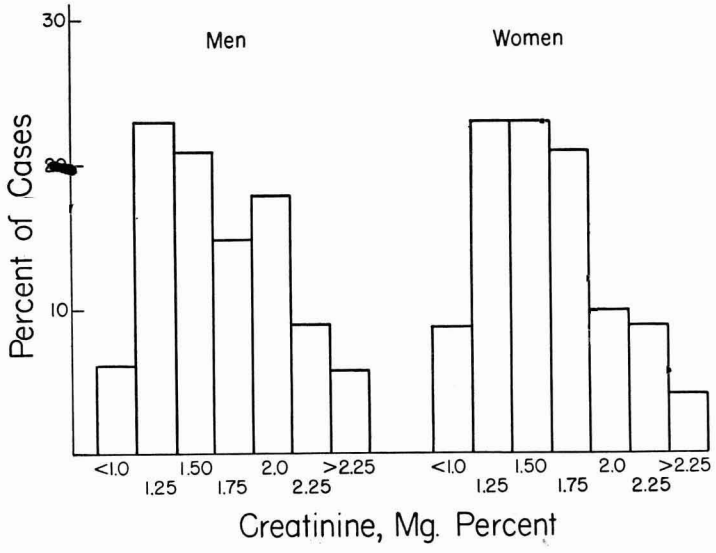

Fig. 6 The distribution of blood creatinine levels.

Uric acid to creatinine ratios

Since blood creatinine levels are more likely to remain constant under varying dietary conditions, as well as in renal impairment, than are uric acid levels, the ratio of these levels might provide an additional index of protein metabolism and renal function. This ratio proved to be close to 2.0 in all groups (table 3). After the age of 75 however a drop in the ratio occurred in both men and women due chiefly to the decrease in uric acid.

Albuminuria

There were 24 men and 23 women whose urines showed some albumin, 16 with traces and 31 with definite precipitates. No

relationship to serum protein level could be detected although 18 of the men and 10 of the women had 6.5 gm % or more total serum protein. This was a significantly higher proportion of men with this serum protein level than was found in the entire group. The proportion of women with this level and albuminuria was somewhat less than the total average for women. No relationship of albuminuria to nonprotein nitrogen, uric acid or creatinine blood levels was seen in either men or women.

SUMMARY

The total serum protein levels were determined in 573 men and women over 50 years of age and the nonprotein nitrogen, uric acid and creatinine levels in the blood of 255 of these. Seven-day diet records and physical examinations were also recorded.

The average serum protein level was 6.47 ± 0.06 gm% in the men and 6.44 ± 0.05 in the women. The range was 5.2 to 7.4, but about 90% of all values fell between 6.0 and 7.0. These levels are 10 to 15% lower than those usually quoted for young adults. No correlation with protein intake was found nor was there any age or sex difference but there was some positive relationship to hemoglobin levels.

The blood nonprotein nitrogen was greater in the men than in the women, 38 ± 1.4 and 34 ± 1.2, but after 70 years this difference was not significant. In the men but not in the women the nonprotein nitrogen levels were positively correlated with protein intake. The mean levels were about 40% higher than those usually found in young adults. The blood uric acid levels were also higher in men than women but after 70 years the differences were less marked and the levels decreased. The mean values 3.24 ± 0.19 mg% in men and 2.98 ± 0.18 in women were 28% larger than those found by the same method in a group of young men and women, 2.53 and 2.33 mg%. There was no correlation with the fat intake but a small positive correlation with protein intake in the men but not in the women.

The blood creatinine levels were not affected by sex or age. The mean values were 1.53 ± 0.11 and 1.50 ± 0.08 mg% for men and women respectively. No correlation with protein intake could be detected. These levels are 20 to 30% higher than those usually quoted for young adults or for the group of 23 young people examined in this study, 1.27 ± 0.10 and 1.18 ± 0.07 mg% for young men and women respectively. The data suggest greater response of the nitrogen metabolism to dietary protein in older men than in women or gradual failure of kidney function which is more marked in the men.

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