METABOLISM OF PHOSPHORUS^{32} AND MOLYBDENUM^{99} IN RATS RECEIVING HIGH CALCIUM DIETS [1,2]

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The syndrome of molybdenum toxicity in animals includes abnormalities which indicate disturbed phosphorus metabolism. In areas where copper is deficient and molybdenum is excessive in the forage, a condition resembling rickets in young animals and osteomalacia in older animals has been observed. (Britton and Goss, '46; Davis, '50; Cunningham, '50.) Stiffness, broken bones, beaded ribs and enlarged and eroded joints were common. Thomas and Moss ('51) have also observed stiffness, bone involvement and erosion of some joints in young dairy bulls fed molybdenum under experimental conditions. An abnormality of the front legs and a change in the bone structure of rabbits fed excess molybdenum has been reported. (Arrington and Davis, '53; Lindenstruth, '54.)

The results of other experiments which were designed to study phosphorus metabolism in animals receiving molybdenum have indicated an alteration of the normal phosphorus metabolism (Comar et al., '49; Shirley et al., '50, '51). The significant change in phosphorus metabolism which was observed in steers fed molybdenum was observed to a much less extent in rats (Shirley et al., '51). Subsequent studies with rats and

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rabbits have indicated no change in the normal phosphorus metabolism in these species when consuming high levels of molybdenum (Arrington and Davis, '53b).

The cause of the skeletal abnormalities associated with molybdenum toxicity is unknown. Since the phosphorus metabolism appears to be altered in some species and the molybdenum content of bone and other tissues increase under these conditions (Fairhall et al., '45; Kulwich et al., '53), another approach to the study of the possible relationship of phosphorus and molybdenum was considered. If the two elements follow the same path, or if molybdenum combines with or partially replaces phosphorus, then an alteration of the diet which would change the metabolism of phosphorus might also change the behavior of molybdenum. This experiment was designed to study the effect of high dietary calcium upon the metabolism of P^{32} and Mo^{99}.

EXPERIMENTAL

Forty-eight mature rats of the Long-Evans strain were distributed at random into two groups and fed rations which differed only in the calcium content. The basal ration was composed of 3415 gm powdered whole milk (Klim), 3380 gm sucrose, 34 gm NaCl, 0.136 gm $FeSO_4 \cdot 7H_2O$, 0.060 gm $MnSO_4 \cdot 2H_2O$, 0.1 gm $CuSO_4 \cdot 5H_2O$, 0.023 gm thiamine hydrochloride and 0.033 gm pyridoxine hydrochloride. The calcium to phosphorus ratio in the ration was 1.3 to 1 and the intake of calcium and phosphorus on this diet was adequate. The high-calcium diet was prepared by adding $CaCO_3$ to the basal ration so that the calcium to phosphorus ratio was 3 to 1. These diets were fed ad libitum to both groups and vitamin D in the form of calciferol was given weekly.

After at least 5 weeks on these diets, the rats were given an oral dose of P^{32} or Mo^{99} and confined to individual metabolism cages for separation and quantitative collection of the urine and feces. Rats dosed with P^{32} received 5 μc of this isotope and those dosed with Mo^{99} received 15 μc. The P^{32} was in the form of H_3PO_4 in weak HCl and was adjusted

to a pH of 6.5 for dosing. The Mo^{99} was in the form of MoO_3 when received, and was neutralized and taken into solution with NaOH prior to dosing.

Periodic urinary and fecal collections were made. At the end of the metabolism period, animals were sacrificed and tissue samples taken for distribution studies. All samples

TABLE 1

Excretion of P^{32} and Mo^{99} by mature rats receiving normal and high calcium diets

RATION	NO. RATS	HOURS AFTER DOSE	URINE	FECES
			(% of dose)	*(% of dose)*
		P^{32} Excretion		
	11	24	5.31 ± 1.48 [1]	23.49 ± 10.05
Control				
	7	48	8.63 ± 1.01	25.15 ± 5.98
	8	24	0.108 ± 0.101	22.67 ± 13.95
High Ca				
	4	48	0.116 ± 0.014	30.00 [2]
		Mo^{99} Excretion		
	10	6	26.38 ± 10.46	. .
Control	6	12	50.82 ± 16.66	. .
	6	24	58.25 ± 15.98	7.98 [3]
	9	6	24.43 ± 11.99	. .
High Ca	5	12	48.85 ± 16.17	. .
	4	24	55.73 ± 18.86	7.70 [3]

[1] Standard deviation.

[2] Represents fecal excretion from one rat.

[3] Fecal excretions at 24 hours were highly variable in amount and in percentage of dose of excreted Mo^{99}.

were wet ashed with nitric acid and the radioactivity determined with a dipping tube fitted to a standard scaler. Results were calculated as percentage of the total dose which was excreted and the percentage of dose per gram of fresh tissue.

RESULTS AND DISCUSSION

Excretion of P^{32}. Data representing the excretion of P^{32} through the urine and feces by rats on the two diets are presented in table 1. The results demonstrate a significant re-

tention of phosphorus when the calcium intake was high. By 48 hours, the control group had excreted 8.6% of the dose through the urinary tract, while rats which were receiving the high intake of calcium had excreted only 0.1% of the dose by this route at 48 hours. The metabolism period for these rats was not carried beyond 48 hours, but when the period was extended to 112 hours with a limited number of other rats, the rate of urinary excretion was not altered. In this trial, one mature rat and one growing rat on each dietary treatment were dosed orally with P^{32}, and periodic urinary and fecal collections were made for 112 hours after dosing. At this time, the percentage of the dose in the urine was 14.3 for the mature and 9.8 for the young rat on the normal calcium intake and 0.33 and 0.12% respectively for those on the high calcium intake. The decreased urinary excretion of P^{32} from the high calcium intake is also reflected in the increased accumulation in the tissues (table 2).

The excretion of P^{32} by way of the feces in the two groups was not different although there were wide variations in the percentage of the dose which was excreted by individual rats (table 1). At 24 hours and at 48 hours the fecal excretion from the two groups was similar. Although the metabolism period was limited to 48 hours, it appears that absorption of phosphorus was less affected than was deposition and retention. Since a large proportion of absorbed phosphorus is excreted by way of the gastro-intestinal tract, it might be expected that the increased calcium intake would also reduce the amount of P^{32} excreted in the feces. The limited metabolism period probably does not represent a sufficient length of time to reflect a change in this route of endogenous phosphorus excretion.

P^{32} in tissues. The decreased urinary excretion of P^{32} by rats with the high intake of calcium (table 1) was accompanied by an increased accumulation of labeled phosphorus in the tissues (table 2). Values in table 2 represent the percentage of dose per gram of fresh tissue corrected to an average weight of the rats. The amount of P^{32} in both soft tissues and

TABLE 2

Tissue distribution of P^{32} and Mo^{99} in mature rats receiving normal and high calcium diets

RATION	NO. RATS	HOURS AFTER DOSE	PERCENTAGE OF DOSE PER GRAM TISSUE				
			Blood	Liver	Kidney	Muscle	Femur
			P^{32} Distribution				
Control	6	24	0.134 [1]	0.721 ± 0.111	0.534 ± 0.113	0.132 ± 0.033	1.031 ± 0.131
	8	48	0.078 ± 0.011 [2]	0.560 ± 0.19	0.487 ± 0.158	0.170 ± 0.079	0.843 ± 0.378
High Ca	5	24	0.428 ± 0.195	1.299 ± 0.529	0.661 ± 0.083	0.142 ± 0.025	1.122 ± 0.195
	3	48	0.106 ± 0.023	0.760 ± 0.099	0.520 ± 0.048	0.256 ± 0.019	0.888 ± 0.205
			Mo^{99} Distribution				
Control	6	6	0.345 ± 0.094	0.185 ± 0.085	0.457 ± 0.210	0.129 ± 0.028	0.277 ± 0.114
	6	24	0.038 ± 0.023	0.021 ± 0.009	0.054 ± 0.016	0.008 ± 0.0045	0.036 ± 0.010
High Ca	5	6	0.327 ± 0.078	0.144 ± 0.023	0.349 ± 0.108	0.091 ± 0.066	0.243 ± 0.175
	6	24	0.035 ± 0.017	0.021 ± 0.004	0.091 ± 0.046	0.019 ± 0.014	0.058 ± 0.037

[1] Represents blood from one rat.

[2] Standard deviation.

bone was greater when the rats had consumed the high calcium diet, and the differences were apparent at the 24- and 48-hour intervals after dosing. Changes in phosphorus deposition were greatest in liver and blood, followed by kidney, muscle and femur. A greater deposition of P^{32} in bone might be expected, but these rats were mature and the age may account for the relatively low accumulation in the skeletal tissue.

These results present some of the changes in phosphorus metabolism which occur with increased calcium intake and further suggest the necessity for careful control of the calcium-to-phosphorus ratio in any phosphorus balance study.

Excretion of Mo^{99}. The metabolism of Mo^{99} was not altered as was that of P^{32} by the presence of excess calcium in the diet. Data representing urinary and fecal excretion by the two groups are presented in table 1. Within 24 hours after dosing, more than 50% of the Mo^{99} administered had been excreted through the urine and approximately 8% through the feces by the rats on each ration. It appears from the table that the urinary excretion of Mo^{99} by the rats receiving the high calcium intake was less than the control, but the difference is not statistically significant.

Mo^{99} was eliminated largely through the kidney and was very rapidly excreted. Within 6 hours after oral administration, approximately 25% of the dose had been excreted in the urine. At 12 hours, 50% was present in the urine. The excretion of molybdenum by this route and the rapid rate of elimination from the body has also been observed by other investigators (Fairhall et al., '45; Neilands et al., '48; Comar et al., '49).

Mo^{99} in tissues. Values for the corrected percentage dose of Mo^{99} per gram of fresh tissue are recorded in table 2. The amounts of labeled molybdenum in the tissues of rats from the two diets were similar with no consistent differences which may be attributed to changes in the intake of calcium. The pattern of distribution in both groups was unlike that of phosphorus in that kidney and blood predominated in

Mo^{99} accumulation, followed by bone, liver and muscle. A higher content of molybdenum in the kidney should be expected since the urinary system is the chief route of excretion. The rapid excretion of molybdenum is reflected in the difference in amount of Mo^{99} in the tissues at 6 hours and at 24 hours. Considerable variation was observed in the quantity of Mo^{99} in excreta and tissues between individual rats of both groups. Similar individual variations in molybdenum metabolism have been observed by others (Singer, '49).

These results indicate that there were no changes in absorption, retention or excretion of Mo^{99} associated with increased calcium intake.

SUMMARY

The metabolism of P^{32} and Mo^{99} was studied in mature rats which were consuming normal and high calcium diets. A decreased urinary excretion and increased tissue deposition of P^{32} demonstrated a significant retention of phosphorus when the intake of calcium was high. Metabolism of Mo^{99} was not changed by the increase in dietary calcium. The rapid elimination of molybdenum by way of the urine was demonstrated, 50% of the oral dose being excreted by 12 hours.

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THE VALIDITY OF FOLIN'S CONCEPT OF DICHOTOMY IN PROTEIN METABOLISM

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FOUR FIGURES

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The value of a theory in science should not be judged by its validity or otherwise, as ultimately determined, but by the extent to which it harmonizes and explains contemporary knowledge. The value of Folin's ('05) theory of protein metabolism by this criterion has been tremendous. The essential feature of the theory is the concept of dichotomy into the endogenous protein metabolism and the exogenous protein metabolism. The one is constant under normal conditions and pertains to the slow erosion of the nitrogenous constituents of the tissues; the other is variable, being dependent in rate on the protein intake.

The theory accounts adequately for the known facts concerning a minimum requirement of dietary protein for the maintenance of nitrogen equilibrium, in proportion to body size, and for the acceleration of the rate of output of nitrogen in the urine in proportion to the intake of dietary protein and of its nutritive quality. The theory is consistent with the fact that the protein requirement in adult nutrition, regardless of species, expressed as a percentage of the fiber-free, moderate-fat, dry matter required for maintenance of body weight is much the same, namely, 4 to 6%, for proteins of good quality.

The requirement of dietary nitrogen, when largely provided by protein of high nutritional quality, for the maintenance of nitrogen equilibrium is strikingly similar for

different species of monogastric animals, not in proportion to body weight, but in proportion to body surface. Experimental data with reference to this point have been assembled in table 1. From this evidence, including all with which the author is familiar, it appears that mature monogastric animals

TABLE 1

The requirement of dietary nitrogen by mature animals of different species

SPECIES OF ANIMAL	TYPE OF DIETARY PROTEIN	NITROGEN REQUIRED FOR N EQUILIBRIUM	AUTHORITY
		gm/m²/day	
Rat	Whole egg	1.91 [1]	Bricker and Mitchell ('47)
	Milk	2.18 [1]	Bricker and Mitchell ('47)
	Soy flour	3.76 [1]	Bricker and Mitchell ('47)
Dog	Casein	2.70 [2]	Risser ('46)
	Lactalbumin	1.70 [2]	Kade, Phillips and Phillips ('48)
	Casein	2.62 [2]	Kade, Phillips and Phillips ('48)
	Casein and methionine	1.71 [2]	Kade, Phillips and Phillips ('48)
	Blood fibrin	2.16 [2]	Kade, Phillips and Phillips ('48)
	Lactalbumin	3.42 [2]	Melnick and Cowgill ('37)
	Blood serum protein	3.55 [2]	Melnick and Cowgill ('37)
	Casein	4.37 [2]	Melnick and Cowgill ('37)
Man	Mixed plant	2.68 [1]	Hegsted et al. ('46)
	Same plus meat	2.35 [1]	Hegsted et al. ('46)
	Milk	2.03 [1]	Bricker, Mitchell and Kinsman ('45)
	White flour	3.62 [1]	Bricker, Mitchell and Kinsman ('45)
	Soy flour	2.06 [1]	Bricker, Mitchell and Kinsman ('45)
	Mixed diet	2.20 [1]	Bricker, Mitchell and Kinsman ('45)
	High cereal, some meat	2.27 [1]	Bricker et al. ('49)

[1] Truly absorbed N with due regard to metabolic fecal N.
[2] Total N intake.

generally require from 2 to 4 gm of nitrogen from high quality proteins per m² of body surface per day to maintain nitrogen equilibrium.

The minimum endogenous urinary nitrogen is attained under conditions of specific protein inanition after varying periods of subsistence on a near nitrogen-free regime, the time required being proportional to the size of the animal

(Smuts, '35); it remains constant for a period of days if the intake of food energy is adequate and then decreases further, behaving similarly in this respect to the metabolic rate after the withdrawal of food. While many illustrations of this trend in the endogenous urinary nitrogen can be cited, the experiment of Smith ('26) is worthy of particular notice. A man was maintained for 24 days on a diet containing less than 1 gm of nitrogen per day, of which he consumed enough to maintain body weight with only slight fluctuations. The nitrogen output in the urine for a period of 10 days, starting with the third day on this regime, was remarkably constant. The regression line of urinary N on time on test was — 0.0873 with a variance of 0.0025, a t value of — 1.76 and a probability of about 0.1. There is thus no reason to believe that during this interval of time a level of output of urinary nitrogen had not been reached, a statement in the literature to the contrary notwithstanding (Frost, '50). The minimum endogenous urinary nitrogen is not only a reality but it is attainable experimentally in all animals with which an earnest attempt has been made, even with the ruminant animal (Kehar, Mukherjee and Sen, '43; Mukherjee and Mitchell, '51). The minimum endogenous catabolism of nitrogen may be considered to be only one phase of the total basal expenditure of energy and would be expected to vary with it. This has been shown to be true by Terroine and Sorg-Matter ('27) and by Smuts ('35), for mature animals of many species. The latter investigator found a ratio of minimum endogenous urinary nitrogen to basal metabolism of 2 mg to 1 cal. for 6 species of animals; the coefficient of variation of the ratio for the 56 animals upon whom both measurements were made was only 8.12%. In the experiment of Smith above referred to, the basal metabolism of the subject was determined on the 13th day of the experiment and was found to be 1383 cal. per day when the urinary nitrogen on the same day was 2880 mg, the ratio being 2.08 mg per basal calorie.

The output of creatinine, a characteristic constituent of the endogenous catabolism according to Folin, is also closely

related to the basal metabolism (Palmer, Means and Gamble, '14; Smuts, '35), within any one species of animal.

The Folin theory of a dichotomy in protein metabolism is in agreement with mounting evidence of a dichotomy in the total metabolism of organic nutrients in the body secured with radioactive isotopes, S^{35} (Tarver and Morse, '48), tritium (Thompson, '52, '53), and N^{15} (Hoberman, '51). All of these reports, as well as others that might be cited, reveal the existence in the animal body of two categories of organic compounds, one in a dynamic state of metabolism with a short half-life, and the other in a relatively static state with a long half-life. As Thompson ('53) says, in speaking of the latter category: "It follows necessarily that there must exist in the animal a truly endogenous metabolism in which these compounds, at whatever rate they may be turning over in a specific tissue, are being only very slowly re-synthesized or replaced from dietary sources." Using N^{15}-glycine as an indicator in rats on different levels of protein nutrition, Hoberman, from observations of the excretion of N^{15} during a fast of 192 hours following administration of the indicator, obtained results suggesting "that in fasting animals two reservoirs of protein contribute nitrogen to the animo acid pool. One reservoir interacts rapidly with the amino acids of the tissues, while another portion of the body proteins would appear to be susceptible to virtually irreversible hydrolysis."

Tarver and Morse ('48) observed that after the feeding of S^{35}-methionine to rats, the radioactivity during the following 14 days is lost from the active tissues that have avidly absorbed it, so that the isotope concentration throughout the body becomes more or less stabilized at a low level. This phenomenon has also been observed by Shemin and Rittenberg ('44). In commenting on the approach of the isotopic concentration in all tissues to a constant level and the associated slow loss of isotope into the urine, Tarver and Morse ('48) observe: "We fail to see where the results are in dis-

agreement with the concept of endogenous metabolism as formulated by Folin."

The concept of a dichotomy existing in protein metabolism, first pointed out by Folin, thus harmonizes and explains many of the phenomena of protein metabolism up to the present writing. The view that "exogenous and endogenous metabolites are pooled in the living organism" (Bigwood, '52) and are therefore indistinguishable is not consistent with the most recent work with isotopic nitrogen. This work indicates that only a small fraction of the body proteins is involved in the reversible dynamic reactions with animo acids originating in the gastro-intestinal tract (Sprinson and Rittenberg, '49), the existence of which induced Schoenheimer and his colleagues ('39) to deny the possibility of any distinction between endogenous and exogenous metabolism.

On the other hand, the nature of the exogenous metabolism of protein as visualized by Folin must be radically changed, although "the rate of excretion of urinary urea is . . . related to the oxidative metabolism of the proteins whether of tissue or dietary origin" (San Pietro and Rittenberg, '53), as Folin originally supposed. Therefore, in the interpretation of nitrogen balance data, the application of isotope tracer methods of research to the problems of protein metabolism seems to have had little effect either in the evaluation of protein requirements or of protein utilization in metabolism. The light such methods have cast upon the reactions and the quantitative aspects of the intermediary metabolism of proteins and amino acids has been tremendous and revolutionary.

The experiments to be reported in this paper will be concerned mainly with the validity of the conception of an endogenous catabolism of nitrogenous substances independent of the ingestion of dietary protein. The evidence presented is necessarily indirect.

EXPERIMENTAL PROCEDURE

The purpose of the experiments was to determine whether (1) the urinary nitrogen in growing animals is positively

correlated with the absorbed nitrogen within the range from zero to levels exceeding the capacity of the animal to store protein, and (2) the nitrogen balance is linearly related to the absorbed nitrogen within the same limits. If the first relationship is established it means that, under the conditions imposed, the endogenous urinary nitrogen is not decreased by the ingestion of dietary protein. If the second relationship is established it means that a constant fraction of the absorbed nitrogen above the nitrogen output on a nitrogen-free diet is retained in growth, implying strongly that the nitrogen excretion on a nitrogen-free diet continues at a constant level throughout subsequent periods of increasing nitrogen intake. The independence of the endogenous and the exogenous metabolism of nitrogen would thus be indicated.

The data were taken from experiments on growing rats, fed varying levels of whole egg protein, the fecal excretion results of which were reported previously (Mitchell and Bert, '54). These data were supplemented with results secured in similar experiments involving the feeding of graded levels of beef muscle protein. The two series of experiments were conducted according to the same general plan with reference to diets (except for the nature of the protein), initial weights of rats (50 to 70 gm), diets (complete except for protein content which was varied inversely with the carbohydrate content), length of experimental periods (at least 7 days of pre-feeding followed by 7 days of collection), and preservation and analysis of excreta. In the comparison of different levels of protein the rats were carried through as pairs, the two members of each pair receiving the same amounts of their respective diets with reversal periods designed so that each rat received each of the diets being compared.

The egg protein experiments involved 70 growing rats, 250 nitrogen balance periods and 6 levels of protein ranging from practically zero to about 20%. The beef protein experiments involved 40 growing rats, 64 nitrogen balance periods and 4 levels of dietary protein ranging from practically zero to about 11%.

RESULTS AND DISCUSSION

Figure 1 illustrates the regression for both egg protein and beef protein of the urinary nitrogen on the truly absorbed nitrogen, due regard being taken of the metabolic fecal nitrogen; both variates are expressed in milligrams per 100 cm^2 of body surface per day. Each point in the diagram represents the average of three to 6 individual results in the case

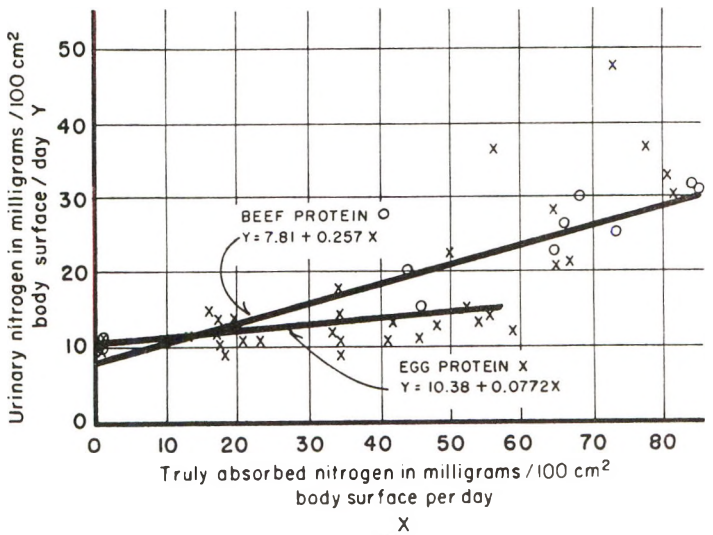

Fig. 1 The regression of urinary nitrogen on absorbed nitrogen in the experiments on egg protein and on beef protein.

of beef protein, and 4 to 12 individual results in the case of egg protein tests.

It will be noted that the data for beef protein exhibit a linear regression up to an absorbed nitrogen level of approximately 85 mg per 100 cm^2 of body surface per day. Beyond this point the results depart from linearity, falling above the regression line (these points have not been entered on the chart). Both the correlation coefficient ($r = 0.95$) and the regression coefficient (0.257) are significantly different from zero at a probability level of considerably less than 1%.

For the egg protein experiments, the range of linear regression is much narrower (zero to about 55 mg of absorbed nitrogen per 100 cm^2 body surface per day), because of the higher nutritional quality of the protein. Both the correlation coefficient ($r = 0.435$) and the regression coefficient, are smaller in the egg protein than in the beef protein experiments, but both are significant at a probability level between 5 and 2%.

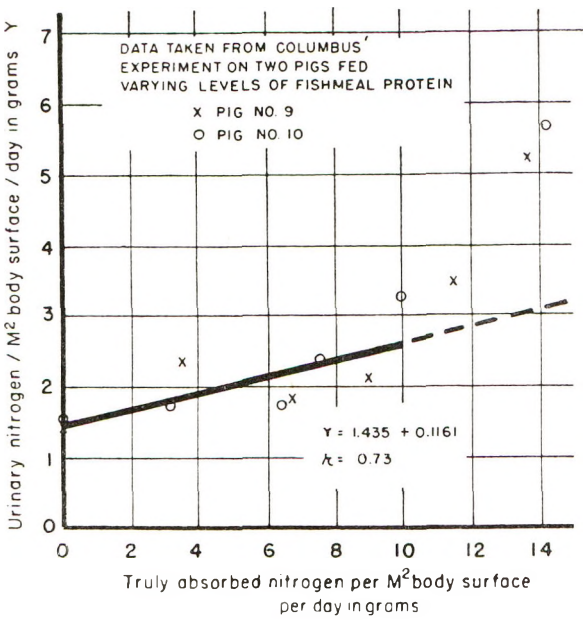

Fig. 2 The regression of urinary nitrogen on absorbed nitrogen for two pigs receiving different levels of fishmeal protein (Columbus, '50).

Similar evidence is presented for the pig by Armstrong and Mitchell ('54), and by appropriate calculations from the findings of Columbus ('50) also on growing pigs (see fig. 2).

These results reveal, in a manner similar to those reported by Burroughs, Burroughs and Mitchell ('40), that under the conditions imposed, the minimum endogenous excretion of urinary nitrogen is not depressed by the addition to the diet of protein mixtures of high nutritional quality. They are consistent with the theory of Folin that the minimum

endogenous nitrogen metabolism is independent of the exogenous nitrogen metabolism.

The reductions that have been reported in the excretion of urinary nitrogen in protein-depleted animals on a nitrogen-low diet by the incorporation in the diet of a high-quality protein or of methionine (Brush, Willman and Swanson, '47; Allison, Anderson and Seeley, '47) may well have resulted from the suppression of an accelerated endogenous nitrogen metabolism. In both investigations the condition of severe protein deficiency coupled with a high-fat intake are conducive to an impairment in liver function that may well involve the metabolism of protein. Li and Freeman ('46) showed that exogenous fat impairs liver function in the protein-deficient dog. The effect of methionine in depressing the accelerated endogenous nitrogen metabolism of the protein-deficient animal (Brush et al., '47; Allison et al., '47; Miller, '44) may be a sequel to its beneficial effect on liver function (Miller, Ross and Whipple, '40). Also the raiding of tissues to supply cystine for feather growth in the hen has been shown by Ackerson and Blish ('26) to accelerate the endogenous nitrogen metabolism by about 50%, an effect that could be obviated by the administration of cystine.

In contrast to the dog and the rat, the human subject on a low-protein diet does not respond to methionine administration by depressing his output of endogenous urinary nitrogen, probably because the latter has not been accelerated by a dominant need for cystine for hair growth. Johnson et al. ('47) in comparing their work with humans with that of Allison et al. ('47) with dogs state: "In this connection, it is interesting to compare the levels of urinary excretion of 1.5 to 1.9 gm $N/m^2/24$ hr., reached in a comparable time with dogs receiving a methionine supplement with the average levels of approximately 1.9 gm $N/m^2/24$ hr. reached by these human subjects without methionine during the last 5 days of the low-protein period." This is further presumptive evidence that methionine will depress the rate of excretion of endogenous urinary nitrogen only when the endogenous met-

abolism has been accelerated above the minimum level by some means, possibly by an impairment of liver function or by an unsatisfied demand for cystine for keratin synthesis.

Figures 3 and 4 demonstrate the linearity of the regression of nitrogen balance on absorbed nitrogen intake in the area of negative balances and, in growing animals, in the area of positive balances up to an intake of absorbed nitrogen

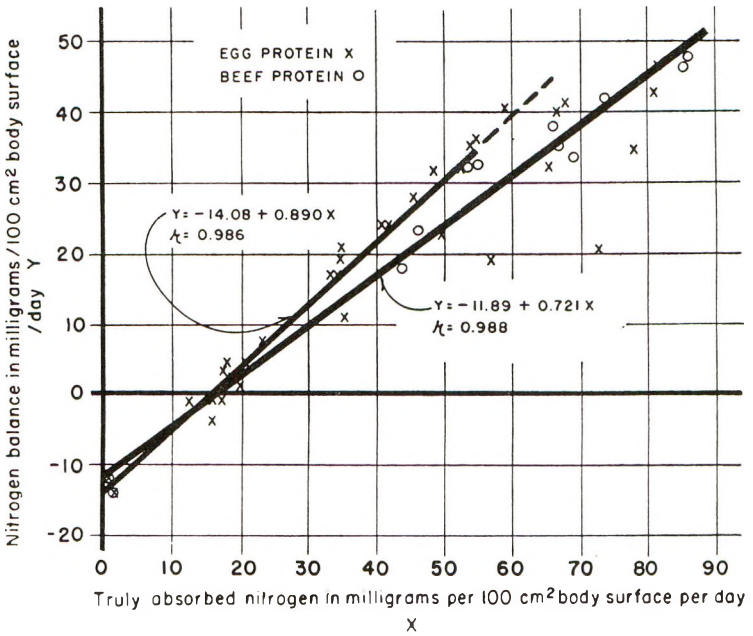

Fig. 3 The regression of nitrogen balance on absorbed nitrogen in the experiments on egg protein and on beef protein.

that does not exceed the capacity of the animal to store nitrogen. This critical intake will be higher the poorer the nutritional quality of the dietary protein. Figure 4 was based upon appropriate calculations from the records of Columbus' ('50) work on pigs. The coefficients of the independent variable in the regression equations may be taken as the average biological values of the respective proteins, when multiplied by 100, since the animals were not severely depleted in their

protein stores. These values are 89 for egg protein, 72 for beef protein and 88 for fishmeal protein. The linearity of the regression testifies to the constancy of utilization of a given protein or protein mixture in the metabolism of growing animals within a range of intake that does not exceed the capacity of the animal to utilize, as fully as the amino

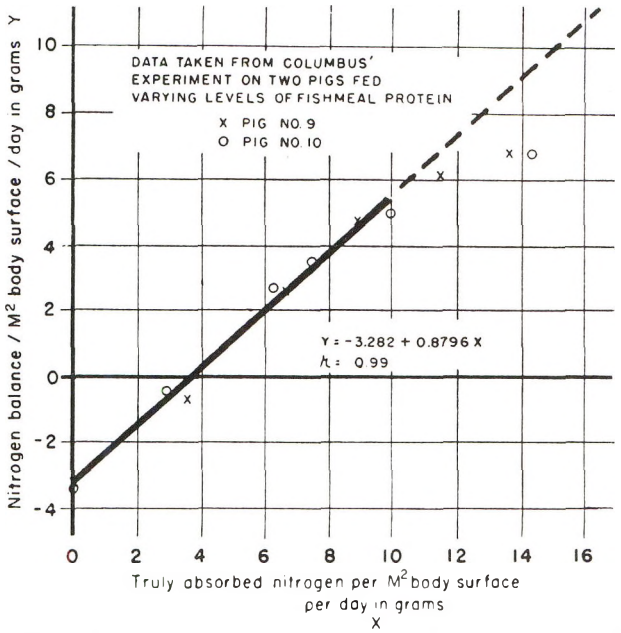

Fig. 4 The regression of nitrogen balance on absorbed nitrogen for two pigs receiving different levels of fishmeal protein (Columbus, '50).

acid composition will permit, all dietary protein at its disposal.

The linearity of the relationship also means that in any one series of experiments the sum of the endogenous urinary nitrogen and the metabolic fecal nitrogen, as measured on a nitrogen-free diet per unit of surface area, is constant, since this sum is equal to the constant term in the regression equation.

These relationships confirm the findings of Allison and co-workers ('45, '46) on dogs. This group has also reported

that the rate of excretion of endogenous urinary nitrogen is a function of the protein stores in the body, being smaller, the smaller the protein stores as measured by the blood plasma protein level. It is an interesting fact, however, that within a wide range of protein stores, and hence of endogenous urinary nitrogen values, the slope of the regression line of nitrogen balance on absorbed nitrogen intake (measuring the biological value or the "nitrogen balance index" of Allison) remains approximately constant, or at least not demonstrably different. Thus, as the rate of the minimum endogenous catabolism of nitrogen shifts, parallel to shifts in the protein stores, the amount of nitrogen required for nitrogen equilibrium shifts in a similar manner, but the indicated efficiency of utilization of dietary nitrogen remains the same. However, in the course of series of experiments such as those described in this paper and in Allison's reports, the change in protein stores is not sufficiently great to disturb appreciably the constancy in the rate of the endogenous nitrogen catabolism.

Hoffman et al. ('48) have found the same situation with reference to human adults, i.e., a high correlation between the minimum endogenous urinary output of nitrogen and the nitrogen intake at equilibrium ($r = 0.86$), and an insignificant correlation between the endogenous urinary nitrogen and the nitrogen balance index ($r = 0.19$). Of the same significance are the findings of Melnick and Cowgill ('37) in their nitrogen balance studies with dogs that the regression lines of nitrogen balance on percentage of protein calories in the diet are essentially parallel, while the minimum protein requirements vary in a manner determined by the extrapolated nitrogen balance at zero intake of nitrogen.

It may be concluded that the concept of dichotomy in protein metabolism, announced by Folin in 1905, explains many of the facts of protein metabolism and is inconsistent with none. Its validity has been established by the evidence presented (or cited) in this paper insofar as evidence can establish a concept not at present susceptible to direct experimental

approach. Recent work with isotope tracers has clarified the nature of the endogenous metabolism and established more firmly its essential constancy. It has afforded an entirely new insight into the exogenous protein metabolism, which is revealed as a dynamic rather than a static activity of the tissues. It is nevertheless a function of the protein intake and may be regarded as truly now as 50 years ago a measure of protein oxidation in the body.

SUMMARY AND CONCLUSIONS

Evidence has been presented from experiments on growing rats and growing pigs receiving different dietary proteins of high nutritional value at different levels of intake to the effect that (1) the urinary nitrogen output of a normal animal on a practically nitrogen-free diet is not necessarily depressed by the ingestion of dietary protein or dietary methionine. Under certain conditions, apparently associated with an accelerated endogenous nitrogen metabolism, such a depression has been reported. It also shows that (2) a constant fraction of the absorbed nitrogen above the nitrogen output on a nitrogen-free diet is retained in the body of the growing animal, implying strongly that the endogenous output of urinary nitrogen continues at a constant level throughout subsequent periods of increasing protein intake. The experimental evidence presented, or cited, in this paper, establishes clearly the validity of Folin's concept of a dichotomy in protein metabolism into endogenous and exogenous types. No well-demonstrated findings in the area of protein metabolism have been found contradictory to this concept; on the contrary many are best explained on this basis. Recent research with isotope tracers has clarified the nature of these dichotomic entities without destroying their identities.

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THE NATURE OF THE CREATINURIA OF NUTRITIONAL MUSCULAR DYSTROPHY IN THE RAT [1]

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ONE FIGURE

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Creatinuria is a cardinal sign of nutritional muscular dystrophy resulting from vitamin E deficiency in the rabbit (Mackenzie and McCollum, '40), and is associated with a decreased concentration of muscle creatine (Goettsch and Brown, '32). Rather simple calculations show that this creatinuria must reflect an accelerated rate of creatine synthesis (Heinrich and Mattill, '49). This condition of increased creatine excretion and reduced muscle creatine might result from an inability of the animal to remove creatine from the bloodstream and incorporate it into muscle tissue, or it might result from an inability of the muscle to retain creatine after its incorporation. This problem can best be approached by the use of isotopically labeled precursors of creatine. Following the injection of such compounds, the specific activities of excreted creatine and creatinine should indicate which of the above mentioned possibilities exist. If the first suggestion is correct the specific activity of creatine excreted by the deficient animals should be much higher than the creatinine and the specific activity of creatinine would not be expected to be elevated when compared to normal animals. In con-

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trast, if the lesion is the result of an impaired ability of the muscle to retain creatine, the excreted creatine should have the same specific activity as the creatinine since it would be diluted with muscle creatine which is the precursor of creatinine. Also in the latter condition, the turnover rate of muscle creatine would be higher and consequently the specific activity of excreted creatinine should be higher in the deficient animals.

Such a study can best be conducted with rats since smaller quantities of the isotopically labeled precursor are required and more animals can be conveniently employed. In the present experiments, nutritional muscular dystrophy was produced in rats by the feeding of a diet deficient in both vitamins E and B_6. It was pointed out by Mackenzie ('53) that under such conditions rats develop the typical microscopic muscle lesions which are characteristic of nutritional muscular dystrophy. Also it has been shown that the combined deficiency results in granulocytosis (Dinning et al., '54) which is seen in rabbits deficient in vitamin E (Dinning, '52). Data to be presented in the present report show that the doubly deficient animals excrete large quantities of creatine. Unpublished data from this laboratory show that rats deficient in both vitamins E and B_6 excrete increased amounts of allantoin and exhibit elevated liver xanthine oxidase activities. Both of the latter conditions are characteristic of vitamin E-deficient rabbits (Young and Dinning, '51; Dinning, '53). It then seems from a consideration of all available data that rats deficient in both vitamins E and B_6 suffer from nutritional muscular dystrophy similar to that observed in rabbits deficient in vitamin E.

METHODS

Weanling Sprague-Dawley rats of both sexes were divided into 4 groups and given the same basal diet and supplements described previously (Dinning et al., '54). After 70 days of feeding rats were taken for the experiments which continued through the 90th day of feeding. The creatine precursors

used were sodium C^{14} formate (1 Mc/mM) [2] and C-14 methyl labeled choline chloride (1.8 Mc/mM). The dosage employed was 10 μc per 100 gm of body weight. Following the injections the rats were placed in metabolism cages for 16-hour urine collection. All the data are in terms of the 16-hour time period. An aliquot of the urine was used for chemical determination of creatine and creatinine by the Jaffe reaction. Creatinine and total creatinine were isolated as the zinc chloride salt

TABLE 1

The influence of vitamins E and B_6 on the excretion of creatine and creatinine and on the incorporation of C^{14} methyl choline into creatinine by rats

SUPPLEMENT	NO. OF RATS	AV. BODY WEIGHT	CREATINE EXCRETION	CREATININE EXCRETION	CREATININE SPECIFIC ACTIVITY
		gm	*mg/100 gm*	*mg/100 gm*	*C.P.M./μM* [1]
None	6	90	4.45	2.04	159
Vitamin E	3	91	1.18	2.87	72
Vitamin B_6	3	228	1.82	2.20	69
Vitamins E and B_6	5	221	0.17	2.70	35

[1] Counts per minute per micromole.

after the addition of carrier and purified to constant radioactivity. Creatine activity was determined by difference between preformed creatinine and total creatinine. Creatine was converted to creatinine by autoclaving in the presence of HCl. The results are expressed as counts per minute per micromole of original creatine and creatinine (concentration before addition of carrier).

The data were analyzed by the analysis of variance method (Snedecor, '46) and a summary of the statistical findings is given in table 3.

RESULTS AND DISCUSSION

Data obtained from the choline experiments are given in table 1. Vitamin B_6 deficiency resulted in impaired growth of the animals whereas in these experiments no growth depression resulted from vitamin E deficiency. Rats deficient

[2] Millicuries per millimole.

in both vitamins excreted large quantities of creatine. This creatinuria was reduced when the animals were given either vitamin E or B_6, the combination of the two was more effective than either of them singly. The addition of vitamin E to the diet increased creatinine excretion regardless of the supply of vitamin B_6. The specific activity of creatinine excreted following the injection of methyl-labeled choline

TABLE 2

The influence of vitamins E and B_6 on the excretion of creatine and creatinine and on the incorporation of C^{14} formate into creatinine and creatine by rats

SUPPLEMENT	NO. OF RATS	AV. BODY WEIGHT	CREATINE EXCRETION	CREATININE EXCRETION	CREATININE SPECIFIC ACTIVITY	CREATINE SPECIFIC ACTIVITY
		gm.	*mg/100 gm*	*mg/100 gm*	*C.P.M./μM* [1]	*C.P.M./μM* [1]
None	6	85	4.38	2.11	103	91
Vitamin E	3	88	0.86	2.48	68	..
Vitamin B_6	3	285	1.22	2.06	81	..
Vitamins E and B_6	5	291	0.04	2.24	51	..

[1] Counts per minute per micromole.

TABLE 3

Results of analysis of variance

MEASUREMENT	EFFECT OF:	
	Vitamin E	Vitamin B_6
	P	P
Creatine excretion	< 0.01	< 0.01
Creatinine excretion	< 0.01	> 0.05
Creatinine specific activity (formate exp.)	< 0.01	< 0.05
Creatinine specific activity (choline exp.)	< 0.05	< 0.05

was greatly elevated when the animals were deficient in both vitamins E and B_6. It is noteworthy that the effects of the various dietary treatments on the specific activity of urinary creatinine paralleled the effect on the quantity of creatine excreted.

Data from the experiments in which sodium C^{14} formate was injected are presented in table 2. The effects of the dietary supplements on body weights, creatine and creatinine

excretion were quite similar to those obtained in the choline experiments. With formate as the precursor the specific activity of urinary creatinine was elevated in the doubly deficient animals and again seemed to be related to the quantity of creatine excreted. In these experiments the specific activity of urinary creatine excreted by the animals deficient

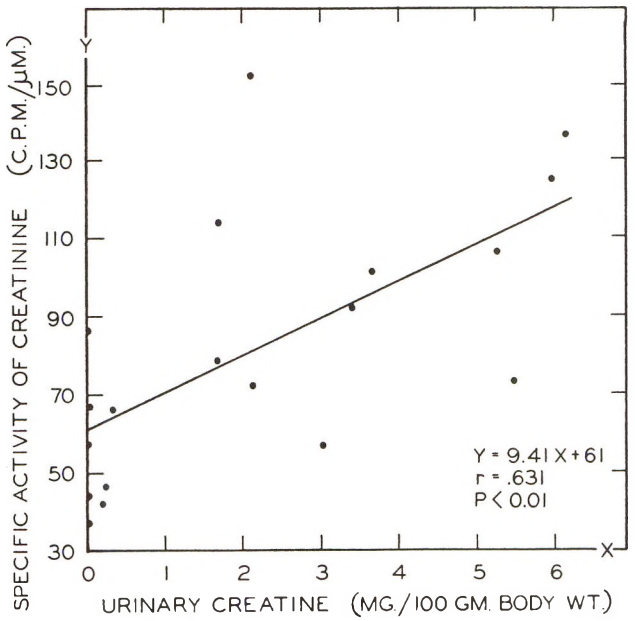

Fig. 1 The relationship between creatine excretion and specific activity of creatinine following the injection of sodium C^{14} formate into rats.

in both vitamins was determined. The value is not significantly different from the specific activity of creatinine excreted by the same rats.

In figure 1 the excretion of creatine is plotted against the specific activity of urinary creatinine. This figure includes data from all rats used in the formate experiments. There was a highly significant correlation coefficient.

The results obtained in these experiments show that the specific activity of creatinine excreted following the injection of either of the radioactive precursors was higher in

rats suffering from nutritional muscular dystrophy. Since urinary creatinine is derived from muscle creatine it follows that more newly synthesized creatine was deposited in the muscle of the deficient animals during the 16 hours following the injections. Since neither the concentration of muscle creatine nor the quantity of creatinine excreted is increased in animals with nutritional muscular dystrophy the extra creatine which was deposited in muscles must have been excreted as such in the urine. This consideration leads to the suggestion that the creatine excreted by rats suffering from nutritional muscular dystrophy is the result of an inability of the muscle to retain creatine. This suggestion receives support from the observation that creatine excreted by these animals exhibited the same specific activity as did the creatinine indicating that both were derived from the same pool. The elevated rate of creatine synthesis in nutritional muscular dystrophy may be a compensatory reaction to the inability of the muscle to retain creatine.

SUMMARY

Nutritional muscular dystrophy was induced in rats by the feeding of a diet deficient in both vitamins E and B_6. Other groups of rats received this basal diet supplemented with vitamins E and B_6 singly and in combination. The rats were injected with either sodium C^{14} formate or C^{14} methyl-labeled choline and the specific activities of excreted creatinine and creatine were determined. Results were similar with both of the labeled creatine presursors. The animals deficient in both vitamins E and B_6 excreted large amounts of creatine. The specific activity of creatinine excreted following the injection of either radioactive precursor was elevated in the rats deficient in both vitamins. The specific activity of creatine excreted by the doubly deficient animals following the administration of sodium C^{14} formate exhibited the same specific activity as did the creatinine. It is concluded that the creatinuria which accompanies nutritional muscular dystrophy

in the rat is the result of an inability of the muscle to retain creatine after its incorporation.

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ASCORBIC ACID UTILIZATION BY WOMEN

RESPONSE OF SERUM LEVEL AND NIGHT URINARY EXCRETION TO INCREASING LEVELS OF INTAKE [1]

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ONE FIGURE

(Received for publication August 18, 1954)

Studies of human ascorbic acid utilization and need have recently been reviewed by Lowry ('52) and Whitacre ('53). In observing responses of human blood values to various controlled levels of ascorbic acid intake, Lowry et al. ('46) found that intakes of 8 and 23 mg per day both resulted in a serum ascorbic acid level of 0.2 mg per 100 ml and a body tissue saturation of 50 to 60% (as estimated from white cell ascorbic acid content and retention of the vitamin following massive doses). An intake of 78 mg per day (whether entirely from food or largely as the pure vitamin) resulted in an average serum level of 0.76 mg and approximately 90% total body saturation. The authors point out the important public health question as to what degree of tissue saturation is necessary for optimal ascorbic acid nutriture. Their findings suggest that a degree of saturation of even as much as 60 to 90% of maximum might still handicap certain body tissues in their constant efforts to combat injury due to mechanical

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[2] The authors wish to express appreciation to Walter D. Foster of West Virginia University, regional biometrician, for statistical examination of the data.

trauma and to bacterial invasion. Since the reviews of Lowry and Whitacre, Davey et al. ('52), Steele et al. ('52, '53), Lutz et al. ('54), and others have reported studies of blood ascorbic acid values in response to various levels of intake of the vitamin in an effort to determine the level of intake for maximum saturation and optimum nutritional state.

The present study is a part of the Cooperative Nutritional Status Project being conducted by the Agricultural Experiment Stations of 11 northeastern states to determine the relationship of nutrient intake to nutritional status in human subjects on various controlled levels of intake. Ascorbic acid is the nutrient under investigation at the Storrs Experiment Station. The responses of serum ascorbic acid and of 8-hour night urinary excretion to various controlled levels of intake of the vitamin were studied for a period of 5 months on 20 women. The plans for this project were based in part on the findings of the previous cooperative nutrition project of the northeastern states (Northeast Region, '51; Babcock et al., '52; Tucker et al., '52; Young et al., '52; Clayton et al., '53).

PROCEDURE

Subjects. Twenty women, patients at a state training school and hospital,[3] served as subjects during the winter and spring of 1952–1953. All were in good physical health and were cooperative throughout the study. They ranged in age from 27 to 63 with only two over 40 years of age.

Dietary ascorbic acid. Throughout the 5 months of the study the subjects were on the regular institution diet except

[3] Mansfield State Training School and Hospital, Mansfield Depot, Connecticut. Thanks are extended to the medical and dietary staffs of the school as follows: To Dr. Gail F. Moxon, M.D., and Dr. Harriet Bixby, M.D., resident doctors, for the physical examinations and for taking the venous blood samples; to Dr. Luke Grotano, D.D.S., resident dentist, for the dental examinations; to Mrs. Pauline Duckett, chief dietitian, and to the dietary staff of the women's dining room, for cooperation in the collection of dietary data and of food samples; to the 20 mentally retarded women who served so cheerfully and cooperatively as subjects; and to Dr. Neil A. Dayton, M.D., Superintendent of the Training School, for making the institution available for the study and for his continued interest and encouragement in research work.

for the omission of citrus fruits and tomatoes in order to have a somewhat restricted and relatively constant dietary ascorbic acid level. Other fruits and vegetables were always served to the subjects in place of citrus fruits and tomatoes when the latter were on the institution menu.

The food intake of each subject was recorded on 14 scattered days each month including the 4 days just previous to the collection of the blood samples at the end of each month. In these 4-day periods samples of individual foods were collected in the dining hall and were analyzed for ascorbic acid. In preparing food samples for analysis, 50 gm of food were blended with 350 ml of 5% metaphosphoric-10% acetic acid and filtered. The filtrate was assayed for total ascorbic acid by the 2,4-dinitrophenylhydrazine method of Roe and Kuether ('43) using norit oxidation. Daily individual ascorbic acid intakes of the subjects were calculated for the 14 days each month using the values obtained in the laboratory.

Ascorbic acid supplementation. After two months on the diet restricted in ascorbic acid, without supplementation, the subjects were selected and the two groups were matched on the basis of serum ascorbic acid levels. During the next three months one group received ascorbic acid supplementation; the other group served as a control. The experimental group received 25 mg of ascorbic acid supplement per day (given in one oral dose, at 4:30 P.M. just before the evening meal) during the third month of the study, 50 mg per day during the 4th month, and 100 mg during the 5th month. The control subjects received placeboes daily.

Serum ascorbic acid. Venous blood samples for serum ascorbic acid determinations were taken once at the beginning of the study, after one month and after two months on the restricted diet, and at the end of each month on the various levels of supplementation. The samples were always taken at 10:00 A.M., three to 4 hours after a vitamin C-free breakfast. Preparation and analysis of the serum for ascorbic acid were carried out according to the procedure as outlined in the Northeast Regional Publication on Techniques ('51).

Ascorbic acid excretion. Eight-hour urine collections were made twice on each subject near the end of each supplemental period. Each collection consisted of the urine formed between 9:30 P.M., the retiring hour (5 hours after the evening meal), and 5:30 A.M., the rising hour. The urine was acidified upon collection and was analyzed for total ascorbic acid by the Roe and Kuether method ('43), using 4% trichloroacetic acid for dilution and norit for oxidation. The above 8-hour collection procedure was used because it was impossible to obtain 24-hour urine collections. Since Todhunter et al. ('42) found in their subjects that the plasma vitamin C level reached its peak between one and two hours and returned to the previous level in about 4 hours, following single doses of 50 or 100 mg of the vitamin, the night urine might be considered a measure of the basal excretion level, giving some indication of degree of body saturation.

Physical examinations. All subjects received complete physical and dental examinations at the beginning and at the end of the study. Presence or absence of those physical signs thought to be associated with ascorbic acid deficiency were carefully noted and recorded.

RESULTS

Dietary ascorbic acid. Individual daily dietary ascorbic acid intakes were found to average 45 mg for the 56 scattered days on which food intake was recorded. The average daily intake for the 4-day periods each month just preceding the blood sampling days was 43 mg.

Serum ascorbic acid. Individual serum ascorbic acid levels at the beginning of the study (just before the restricted diet was begun) averaged 1.02 mg per 100 ml for the experimental subjects and 0.93 mg for the control subjects. At the end of the two months on the restricted ascorbic acid diet without supplementation their average serum levels had dropped to 0.42 and 0.43 mg respectively. The average serum value for the experimental subjects reached the maximum level of 1.49 mg following the month on the 50 mg daily supplement.

In the control group the average serum level dropped, after 5 months on the restricted ascorbic acid diet, to 0.13 mg. These values are shown graphically in figure 1. The difference between these average values was highly significant ($P \leqq 0.01$).

Ascorbic acid excretion. An indication of ascorbic acid utilization and need may be noted from the urinary excretion

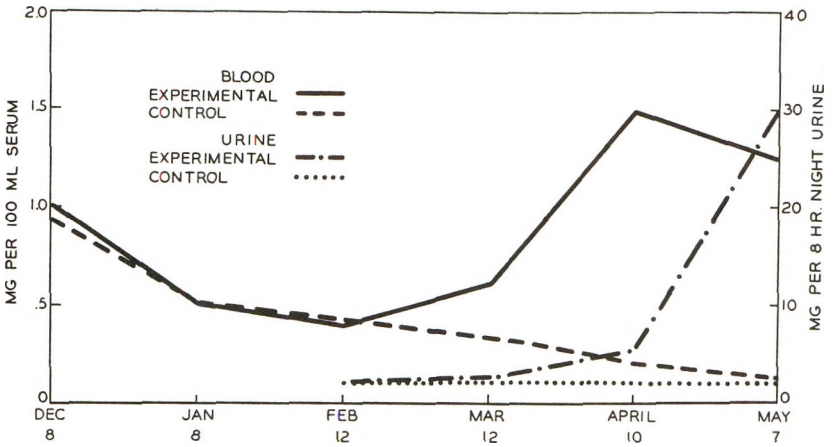

Fig. 1 Average values for ascorbic acid in blood serum and in 8-hour night urine samples at monthly intervals for 10 experimental and 10 control subjects receiving an average dietary intake of 45 mg ascorbic acid per day. All subjects received no ascorbic acid supplement from December 8 to February 12, when the 25 mg daily supplement was begun for the experimental subjects. The 50 mg daily supplement was begun on March 12 and the 100 mg daily supplement on April 10.

rate (in the 8-hour night urine) on the various levels of supplementation. There was but a slight increase in excretion following the addition of the 25-mg supplement. (See fig. 1.) The excretion doubled as a result of the 50-mg supplement but was still only about 5 mg. It increased to about 30 mg after the month on the 100-mg supplement. The control group maintained a uniformly low level of excretion throughout the period. This difference in rate of excretion between the two groups, after the month on the 100-mg daily supplement, was highly significant ($P \leqq 0.01$).

Physical findings. At the end of the 5-month experiment no outstanding changes were observed, in either group of subjects, in the incidence or severity of the few minor physical signs that had been noted in a small proportion of subjects in both groups at the beginning of the study. The period of time involved was seemingly too short and the levels of intake not sufficiently extreme to produce any noticeable changes.

DISCUSSION

Serum levels did not reach a maximum level after one month on a daily ascorbic acid intake of 70 mg (45 mg from food plus a 25-mg supplement of the pure vitamin) although a significant rise was noted. The maximum average serum level was reached after the month on an intake of 95 mg per day (45 mg from food plus a 50-mg supplement). The slight drop in the average serum level following the month on the 100-mg supplement is not significant. The delayed rise in excretion rate, as compared with the earlier rise in serum values with increasing levels of supplementation, may be due to the use of the 8-hour night urine sample in place of the customary 24-hour collection. However, since the blood sample was taken under relatively fasting conditions, the use of the 8-hour night urine sample, collected when the rate of urinary excretion of the vitamin was at its lowest, may serve as a satisfactory biological measurement. The high rate of excretion of the vitamin while on the 100-mg supplement might have been lower if the supplement had been given earlier in the day or in divided doses.

The reaction of the control group throughout the period is of interest. An average daily intake of 45 mg of ascorbic acid was not sufficient to maintain a constant serum level during the 5 months. The serum values dropped gradually, with a slight leveling off during the last month. The urinary excretion of the control group remained at a low and constant level throughout the study.

SUMMARY

1. Two groups of women (10 experimental subjects and 10 controls) were studied regarding their response to various levels of ascorbic acid intake. The experimental subjects, after a two-month period without vitamin supplementation, received 25, 50, and 100 mg per day, each for one month, while on a diet restricted in ascorbic acid (citrus fruits and tomatoes omitted) which provided 45 mg per day. The control subjects were on the same diet, without supplementation.

2. The average serum ascorbic acid level for the 20 subjects fell from 0.97 to 0.42 mg per 100 ml during the two-month period on the restricted ascorbic acid intake. The maximum average serum level of 1.49 mg was reached in the experimental subjects following the month on the 50-mg daily supplement. The average serum ascorbic acid level of the control group dropped to 0.13 mg at the end of the 5 months on the restricted diet.

3. The average value for total ascorbic acid in the 8-hour night urine increased very slightly in the experimental subjects after the 25-mg and the 50-mg daily vitamin supplementation, but did not rise above an average value of 5 mg per subject until following the month on the 100-mg daily supplement, when it increased to an average of 30 mg. The excretion level of the control group remained at about 2 mg throughout the study.

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MANGANESE DEFICIENCY IN THE DUCK[1]

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The essentiality of dietary manganese for the rat was demonstrated by Orent and McCollum ('31) and Kemmerer, Elvehjem and Hart ('31). Wilgus, Norris and Heuser ('36, '37) were first to demonstrate the importance of manganese in poultry nutrition and concluded that perosis in chicks is due to the lack of this element. Weisse et al. ('39) demonstrated that chicks suffering from perosis have lower blood and bone phosphatase activity than normal birds. These investigators also observed that the lowering of phosphatase activity preceded the appearance of slipped tendon. The studies reported here were conducted as part of a series of investigations on the interrelationships between micronutrients and enzyme systems using ducklings as the experimental animal. A number of liver enzymes were investigated in normal and manganese-deficient ducklings and simultaneously the quantitative manganese requirement for normal growth was estimated.

MATERIALS AND METHODS

Day old, white Pekin ducklings of mixed sexes were obtained commercially and on arrival at the laboratory were placed in electrically-heated brooders. The birds were randomized into groups and started on the experimental diets at two days of age. The diets were fed ad libitum and distilled

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water provided at all times. The percentage composition of the basal diet is given in table 1. $MnSO_4 \cdot H_2O$ was added to the basal diet to provide varying amounts of manganese ranging from 0 to 78 mg per kilogram of diet. Since the growth rate of the duckling is extremely rapid, the experiments were usually terminated after three weeks.

The manganese content of the basal diet was determined colorimetrically by the general procedure of the Association

TABLE 1

Composition of the basal diet

CONSTITUENTS		VITAMINS PER 100 GM	
	%		mg
Casein	25.0	Thiamine HCl	0.6
Glucose	58.35	Riboflavin	0.6
Gelatin	5.0	Calcium pantothenate	2.5
DL-Methionine	0.3	Nicotinic acid	7.0
Corn oil	4.0	Pyridoxine HCl	7.0
Salt mixture [1]	6.0	Folic acid	0.25
Choline chloride	0.25	Menadione	0.5
Inositol	0.10	Biotin	0.03
Vitamins in corn oil [2]	1.0	Vitamin B_{12}	0.005

[1] The salt mixture, essentially that of Hegsted et al. ('41), had the following composition in grams: $CaCO_3$, 300; K_2HPO_4, 325; $CaHPO_4 \cdot 2H_2O$, 75; NaCl, 168; $Fe(C_6H_5O_7)_2 \cdot 6H_2O$, 28; $MgSO_4 \cdot 7H_2O$, 100; KI, 0.8; $ZnCO_3$, 0.25; and $CuSO_4 \cdot 5H_2O$, 0.3.

[2] The mixture provided 4,000 I.U. vitamin A, 800 I.U. vitamin D, and 2 mg alpha-tocopherol per 100 gm of diet.

of Official Agricultural Chemists ('50) and was found to be about 1 mg per kilogram of diet. The desired levels of manganese were obtained, therefore, from the added manganese sulfate.

In the experiments involving enzyme and vitamin assays, one group of birds was fed the basal diet, while a second group was maintained on the same diet supplemented with 78 mg of manganese per kilogram of diet. The tissues used for assays were obtained as follows: the birds were decapitated, the tissues removed rapidly and chilled. The tis-

sues were homogenized either in a Ten Brock glass homogenizer or in a Waring Blendor with 10 times their weight of either cold water or 0.1 N phosphate buffer, pH 7.2, depending upon the assays to be run.

Diphosphopyridine nucleotidase (DPNase) was measured using the cyanide addition reaction of Colowick, Kaplan and Ciotti ('51). Activities are expressed as micromoles of DPN split in 7.5 minutes per milligram of protein at 37°C. Alkaline phosphatase was determined using the method of Bessey, Lowry and Brock ('46) in which the breakdown of *p*-nitrophenylphosphate to *p*-nitrophenol is measured. Activities are expressed as micromoles of phosphate released in 30 minutes per milligram of protein. Cytochrome oxidase was assayed by the spectrophotometric method of Smith and Stotz ('49). Reaction rates are expressed as the increase in log Io/I per minute per milligram of protein. Catalase activity was estimated at 37°C. by the perborate method of Feinstein ('49) and activities are expressed as milli-equivalents of perborate degraded in 5 minutes per milligram of protein. Isocitric dehydrogenase activity was measured by the spectrophotometric procedure of Grafflin and Ochoa ('50). Activities are expressed as the increase in log Io/I per minute per milligram of protein. The protein content of the homogenates was determined using the biuret procedure of Robinson and Hogden ('40).

For the assay of riboflavin and niacin, animals were sacrificed by decapitation, the livers removed and homogenized in a Waring blendor. Aliquots were autoclaved in 0.1 N HCl to liberate the bound forms of the vitamins. For pantothenic acid assays, the livers were removed rapidly, immediately plunged into boiling water, and macerated in the blendor. The vitamins were assayed by standard microbiological procedures. Pantothenic acid was assayed for both bound and free forms. The method of Novelli, Kaplan and Lipmann ('49) was employed for the release of the acid from coenzyme A.

RESULTS

Growth

The weight responses of ducklings fed the basal diet supplemented with various levels of manganese are given in table 2. Growth was markedly reduced in the groups fed the unsupplemented basal diet. With the basal diet the mean weights after three weeks for three experiments were 685, 632 and 712 gm as compared to 925, 951 and 936 gm for the ducklings fed 40 mg of manganese per kilogram of diet. At a dietary level of 20 mg of manganese per kilogram of diet almost maximum weight responses were obtained. Although

TABLE 2

Weight responses of ducklings after three weeks on graded levels of manganese

LEVEL OF Mn ADDED	WEIGHTS ± STANDARD ERROR OF MEANS			
	1 7 Ducklings per group	2 [1] 8 Ducklings per group	3 7 Ducklings per group	4 8 Ducklings per group
mg/kg	*gm*	*gm*	*gm*	*gm*
0	685 ± 15	479 ± 21	692 ± 45	712 ± 21
20	852 ± 29	506 ± 30	929 ± 48	890 ± 20
40	988 ± 27	568 ± 37	951 ± 42	936 ± 12

[1] Experiment terminated after two weeks.

there was no statistical difference in the weight responses at 20 and 40 mg of manganese per kilogram of diet, the higher level of manganese in each trial gave a slightly better response. Since an occasional case of perosis was observed at the 20 mg level it would appear that 40 mg of manganese per kilogram of diet would better approximate the requirement for growth and normal development. In some experiments the level of manganese was increased to 78 mg per kilogram of diet but weight responses were no greater than at 40 mg. Wilgus, Norris and Heuser ('37) were able to prevent perosis in chicks almost completely by the addition of 0.0025 to 0.015% of manganese to a diet containing 0.0010% of the element. Insko, Lyons and Martin ('38) in their studies found that 0.0030% of manganese added to a diet containing 0.0005%

was highly effective in preventing the perosis syndrome in chicks. The Committee on Animal Nutrition of the National Research Council ('54) recommends an allowance of about 50 mg of manganese per kilogram of diet for starting chicks and poults. Our results indicate that this value would also be satisfactory for normal growth and development of the duckling.

Enzyme pattern in normal and deficient state

Ducklings fed the manganese-deficient basal diet and the basal diet supplemented with 78 mg of manganese per kilogram of diet were sacrificed and the livers assayed for several

TABLE 3

Effect of manganese on the activities of liver enzymes in normal and deficient ducks

NUTRITIONAL STATE	DPNase	ALKALINE PHOSPHATASE [1]	CYTOCHROME OXIDASE	CATALASE	ISOCITRIC DEHYDROGENASE
	μM/7.5 min./mg P	*μM Phosphate/30 min./mg P*	*ΔOD/min./mg P*	*m.eq. H_2O_2/5 min./mg P*	*ΔOD/min./mg P*
Control	0.049 ± .021	0.43 ± .03	1.99 ± .13	1.69 ± .05	0.057 ± .007
Manganese deficient	0.048 ± .005	0.24 ± .02 [2]	1.88 ± .12	1.96 ± .10	0.075 ± .012

[1] Means of 15 assays.

[2] Significant at 1%.

enzymes. The mean enzyme activity values for 5 assays using a pooled sample of 5 livers for each assay are presented in table 3. Of the enzymes measured, DPNase, cytochrome oxidase, catalase, and isocitric dehydrogenase activities were not significantly altered in the manganese-deficient liver homogenates. Alkaline phosphatase activity, on the contrary, was reduced. Control liver homogenates had an activity of 0.43 μM of phosphate released per 30 minutes per milligram of protein whereas homogenates from manganese-deficient birds had an activity of 0.24 μM of phosphate released. The difference in liver alkaline phosphatase activities was statistically significant at the 1% level of confidence.

When it was found that the liver alkaline phosphatase activity was altered specifically among a number of enzymes, assays were made on various tissues from control and manganese-deficient ducklings. The tissues studied included kidney, brain, blood and heart. The data for these experiments which are presented in table 4 show the means of 7 assays for experiment 1 and 8 assays for experiment 2 except for plasma which involved only three assays. Of the tissues studied, brain alone did not show a statistically significant decrease in alkaline phosphatase during manganese deficiency.

TABLE 4

Alkaline phosphatase activity in various tissues

EXPERIMENT		ALKALINE PHOSPHATASE μM Phosphate/30 min./mg protein			
		Heart	Kidney	Brain	Plasma
1	Control	0.031 ± .007	3.30 ± .32	0.48 ± .04	1.21 ± .21
	Mn-deficient	0.011 ± .002 [1]	0.95 ± .13 [2]	0.41 ± .05	0.53 ± .07 [1]
2	Control	0.023 ± .002	2.71 ± .25	0.30 ± .04	
	Mn-deficient	0.015 ± .001 [2]	0.91 ± .09 [2]	0.24 ± .01	

[1] Significant at 5%.
[2] Significant at 1%.

Kidney alkaline phosphatase was found to be very markedly altered during the deficiency with values of only 29 and 34% of the controls for the two experiments. Wiesse et al. ('39) have demonstrated lower blood and bone phosphatase activity in manganese-deficient chicks. Similarly, Amdur et al. ('45) demonstrated lowered bone phosphatase in manganese-deficient rats. Rabbits fed a manganese-deficient diet did not show significant differences in either acid or alkaline phosphatase activity of the blood serum, kidney, liver and small intestine (Ellis et al., '47). The present findings of marked reduction in liver, kidney, heart and plasma alkaline phosphatase during manganese deficiency may be due to the

severe deficiency produced in the ducklings thus effecting marked physiological alterations.

The lowered assay values for alkaline phosphatase in various tissues might have been due to one or more causes. Several possibilities were considered: (1) tissues of the manganese deficient duckling might contain an enzyme inhibitor, (2) there might have been a shift in the pH optimum for the phosphatases which were being assayed as alkaline phosphatase, or (3) there was an actual decrease in the amount of alkaline phosphatase in relation to total tissue protein.

TABLE 5

Tests for enzyme inhibitor

EXPERIMENT	ALKALINE PHOSPHATASE ACTIVITY μM Phosphate/30 minutes			
	Control	Mn-deficient	Mixture	Expected average
1	0.286	0.055	0.164	0.170
2	0.297	0.056	0.176	0.177

To study the possibility of an enzyme inhibitor being present, aliquots of liver homogenates from control and manganese-deficient ducklings were mixed in equal proportions, assayed for alkaline phosphatase, and compared to the original homogenates. Data for these experiments are given in table 5. The enzyme activity of control homogenates was not inhibited by the presence of homogenates from deficient birds. This suggest that no enzyme inhibitor was present in the deficient tissue. Determinations were also made of the inorganic phosphate content of the liver homogenates, since phosphate is known to markedly inhibit phosphatase activity. No difference was found between the inorganic phosphate content of the control and manganese-deficient tissues. The pH optimum for alkaline phosphatase was determined, using homogenates from control and manganese-deficient ducklings, but no difference was noted. One other study was made in order to determine whether the same change in alkaline phos-

phatase activity would result if manganese ions were used to activate the enzyme instead of magnesium which was used normally. Liver and kidney homogenates from control and manganese-deficient ducklings were assayed using the standard procedure except for the omission of magnesium and the addition of 10^{-3} manganous ions. Essentially the same results were obtained as when magnesium was used as the activator, that is, there was decreased liver and kidney alkaline phosphatase activity in the deficiency. In 4 assays, livers and kidneys from control ducklings showed alkaline phosphatase activities of 0.63 and 2.35 μM of phosphate released per 30 minutes per milligram of protein respectively. Livers and kidneys from deficient ducklings had mean alkaline phosphatase activities of 0.17 and 0.84 μM of phosphate released per 30 minutes per milligram protein, respectively. From the data presently available, it is concluded that there is an actual decrease in the enzyme content in various tissues during manganese deficiency.

B vitamins in liver

The niacin, riboflavin, and bound and free pantothenic acid content of the duck liver has been reported previously (Van Reen and Pearson, '53). During manganese deficiency there was no significant alteration in the vitamin content of the livers. Control values were comparable with the values reported previously except for a slightly higher value for bound pantothenic acid. The mean values for the controls were, per 100 gm moist weight of liver: niacin 12.6 mg; riboflavin 1.6 mg; bound pantothenic acid 3.8 mg; and free pantothenic acid 0.56 mg.

SUMMARY

1. The effects of a dietary deficiency of manganese were investigated using ducklings as the experimental subjects.

2. Forty milligrams of manganese per kilogram of diet are adequate for normal growth and prevention of perosis.

3. Alkaline phosphatase activity of liver, kidney, heart and plasma was reduced during the deficiency.

4. The decreased enzyme activity could not be correlated with the presence of an inhibitor or an altered pH optimum of alkaline phosphatase.

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DENTAL CARIES IN THE ALBINO RAT ON FLUORIDATED AND DISTILLED WATER

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There is an abundance of evidence to show that the addition of fluoride to drinking water in the amount of approximately 1 p.p.m. affords considerable protection to human teeth against dental caries (cf. Missouri Medicine '54). Experimental studies have shown that the incidence and extent of carious lesions can be greatly reduced also in the albino rat fed a cariogenic diet when fluoride is added to the drinking water (Ginn and Volker, '44; Hodge and Finn, '39; Marcovitch and Stanley, '38; McClure, '41a, '43; McClure and Arnold, '41; Muhler and Day, '50, '51; Muhler, Nebergall and Day, '53). The amount of fluoride added to the water in these experiments ranged from 2 to 100 p.p.m. In the single experiment in which the water contained as little as 2 p.p.m. (McClure and Arnold, '41) the total caries score of the animals drinking distilled water was lower than of those drinking the fluoridated water, but this difference, in the opinion of the authors, was of questionable statistical significance. A significant deposition of fluoride in the body of the rat was observed when the drinking water contained 4 p.p.m. (Marcovitch and Stanley, '38). In none of these experiments was an attempt made to determine the minimum amount of fluoride that must be added to the water to provide some protection against dental caries. From the various reports in the literature, we were led to believe that drinking water as ordinarily fluoridated for hu-

man consumption would most probably have no effect on the occurrence and progress of dental caries in the albino rat. Since we had no exact information on this point we have made it a practice to distil all the water given to our experimental animals since our local water supply has been fluoridated over the past several years. We have found upon analysis that the fluoride is removed by distillation.

The latest figures available indicate that water is now fluoridated in over 1000 communities in 43 states including 10 major cities. As laboratory studies on dental caries in the rat are probably being conducted, or will be conducted in some of these areas, it should be helpful to the investigator to know definitely whether fluoridated water modifies the cariogenicity of an experimental diet.

The study reported in this paper was not undertaken to ascertain the level at which the concentration of fluoride must be present in order to give protection to the rats' teeth against dental caries, but merely to determine whether the teeth of the albino rat are made more resistant to a cariogenic diet when water as ordinarily fluoridated is consumed regularly from the time of weaning.

PROCEDURE

Thirty pairs of litter mate albino rats of the Wistar strain, born of mothers who had been on a stock diet consisting of Purina laboratory chow, were selected at weaning and placed on a diet containing 65% sucrose. This diet, which we have heretofore designated as the Harvard diet (Haldi and Wynn, '52), has been shown to be highly cariogenic. Another experiment was run on the same number of animals and in identically the same way except that the animals were fed a diet which has been found in our laboratories to be less cariogenic than the Harvard diet. Half of the animals in each experiment were given distilled water to drink ad libitum throughout the experiment and their litter mates were supplied with tap water which was fluoridated. We were advised by our local Public Health Officer that fluoride is added, vary-

ing with the season of the year, in amounts from 0.7 to 1 p.p.m. Our experiments were run during the last three months of the year.

In order to accelerate the appearance and development of dental caries, all of the experimental animals were desalivated at weaning according to the procedure described elsewhere (Haldi, Wynn, Shaw and Sognnaes, '53). When they had been on the experiment for 80 days, they were sacrificed and the teeth examined for dental caries under a dissecting microscope. The caries score was derived by the method described in a previous publication (Haldi and Wynn, '52).

TABLE 1

Dental caries in the albino rat on fluoridated and distilled water when fed cariogenic diets

NUMBER OF ANIMALS	DIET	DRINKING WATER	NUMBER OF CARIOUS LESIONS	STANDARD DEVIATION	CARIES SCORE	STANDARD DEVIATION
			Average		*Average*	
30	1	Fluoridated	27	± 1.5	62	± 21.6
30	1	Distilled	26	± 1.6	60	± 22.3
30	2	Fluoridated	21	± 3.9	36	± 18.9
30	2	Distilled	21	± 4.4	37	± 19.9

RESULTS

Although the animals were allowed to eat ad libitum, it was found from the records of food consumption, that there was no significant difference in the amount of food that was eaten by the two groups of animals. The exposure of the teeth of the two groups to the cariogenic diet was therefore practically identical. The average gain in weight of the females on the fluoridated and distilled water was exactly the same while that of the males was respectively, 205 and 200 gm. This small difference proved to be of no statistical significance.

The data on the incidence and extent of caries in the two groups of animals show that the fluoridated drinking water did not afford any protection to the teeth against dental

caries. The number of carious molars and the caries score were practically identical in the animals drinking fluoridated and in those drinking distilled water. The males and females were grouped together in the table as we have found that in the albino rats in our colony there is no sex difference in susceptibility or resistance to dental caries when fed a cariogenic diet.

DISCUSSION

In these experiments no attempt was made to determine whether fluoridated water consumed by the mother would have an effect on the offspring. In laboratory studies on dental caries it is a common practice to select the experimental animals at weaning from litters whose mothers have been fed a stock diet. Purina laboratory chow, which serves as the stock diet in many laboratories, contains approximately 23 p.p.m. (Shaw and Sognnaes, '54). It is highly improbable that water containing 0.7 to 1 p.p.m. would give an added protection to the teeth when the diet itself contains such a relatively large amount of fluoride. Our interest in these experiments was therefore not in the prenatal effect of fluoridated water but on the effect it may have on experimental results when consumed during the experimental period after weaning.

It may be reasonably assumed that desalivation played no part in the results of these experiments. That the protective action of fluoride is not by way of the saliva is indicated by the reduction in the number of cavities and in the extent of carious destruction when fluoride was administered by stomach tube after removal of the salivary glands (Sognnaes, '40). Other experiments on desalivated animals (Cheyne, '40) have also practically eliminated the possibility of fluoride acting through saliva. When radioactive fluoride was given by intraperitoneal and intravenous injection (Volker, Sognnaes and Bibby, '41) the average fluoride secretion in the saliva totaled only one one-thousandth of the injected dose. These results are consistent with the observations of McClure

('41b) who found no difference in the fluoride content of the saliva of children drinking fluoride-containing and fluoride-free water.

CONCLUSIONS

Drinking water containing 0.7 to 1 p.p.m. of fluoride had no protective influence on experimental dental caries in albino rats fed a cariogenic diet.

ACKNOWLEDGMENT

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STUDIES ON VITAMIN METABOLISM IN EMETINE POISONING

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In a previous paper it was reported that rats maintained on a diet low in protein exhibit a diminished tolerance to emetine (Guggenheim and Buechler, '48). However, increasing the protein content of the diet, as well as the administration of extracts of liver, muscle and kidney, vitamin B_{12}, aureomycin and streptomycin were shown to raise the resistance of protein-depleted rats to the level of well nourished animals (Guggenheim and Halevy, '54). It appears, therefore, that nutritional factors play an important rôle in the resistance of rats to this drug. The high toxicity of emetine, which hampers its general and intensive use in spite of its great value in the treatment of amoebic infections, is not well understood in terms of biochemical lesions. However, it may be postulated that emetine causes nutritional and metabolic changes in certain tissues, which ultimately present themselves in the form of toxic symptoms. It was, therefore, decided to study the metabolism of rats during prolonged treatment with this drug. This paper presents our studies on vitamin metabolism.

METHODS AND PROCEDURES

All the rats used were males; after weaning they were put on a diet of the following composition: skim milk powder

[1] The results reported here are part of an investigation carried out in partial fulfillment of the requirements for the Ph.D. degree in the Hebrew University, Jerusalem, Israel.

50, cornstarch 43, olive oil 5, salt mixture 2%. This diet was supplemented with thiamine 0.2, riboflavin 0.2, pyridoxine 0.16, calcium pantothenate 1.2, nicotinic acid 3.0 and choline chloride 60 mg per 100 gm of ration. Each rat received weekly 100 I.U. of vitamin A, if not otherwise stated.

In order to compare the emetine treatment of our rats — as far as possible — with the course of treatment of human amoebiasis, we chose repeated administration of the drug. Each rat received daily 0.05 mg emetine hydrochloride subcutaneously, i.e., about 1 to 2 mg per kilogram body weight.

The general procedure was as follows: The rats were treated daily with 0.05 mg of emetine during a period of 4 weeks. At the end of this period they were sacrificed and their livers examined for vitamin content. In the experiments on B vitamins, the urine was collected during three days at the end of both the second and the 4th week, and the amount of vitamins excreted was determined. Since similar values were obtained for the two collection periods, the figures given in table 1 are the combined results.

Because emetine treatment caused a reduction in food intake, the quantity of food offered to the control animals was restricted to the amount eaten by the experimental rats.

The vitamins studied were determined according to the following methods: Vitamin A in liver and carotene in feces colorimetrically as described previously (Guggenheim, '52); thiamine by the thiochrome method (Association of Vitamin Chemists, '51); cocarboxylase according to Westenbrink and Steyn-Parvé ('50); riboflavin fluorometrically (Association of Vitamin Chemists, '51); nicotinic acid by the brom-cyanide method (Sweeney, '51); N-methyl-nicotinamide according to Huff and Perlzweig ('47); pantothenic acid, biotin and folacin microbiologically with *L. arabinosus* and *S. faecalis,* respectively (Association of Vitamin Chemists, '51); Citrovorum factor (CF) with *L. citrovorum* according to Sauberlich and Baumann ('48); synthetic citrovorum factor (Leucovorin) [2]

[2] Leucovorin was supplied by the Lederle Laboratories Division, American Cyanamid Company, by the courtesy of Dr. T. H. Jukes.

in the form of the hydrated barium salt was used as standard. Ascorbic acid was determined by the 2,6-dichlorophenolindophenol method (Mindlin and Butler, '38). Liver folacin was extracted and assayed as described by Dietrich, Monson, Gwoh and Elvehjem ('52). Ascorbic acid was extracted from liver and adrenals by ice cold metaphosphoric acid (6%), dissolved in water distilled twice from an all glass apparatus.

RESULTS

Food intake and growth. One of the most striking effects of the repeated administration of small doses of emetine in young rats is the reduction in food intake and the accompanying limitation of growth. One hundred and six rats treated with emetine increased their weight during 4 weeks by only 28 ± 2.4 gm in contrast to 55 ± 2.0 gm, the weight increase of 99 pair-fed untreated rats. This difference proved to be highly significant. It may be mentioned that untreated control rats of our laboratory, when given the same diet ad libitum, usually increase their weight by 70 gm in 4 weeks. The food consumption of the treated rats was as low as 6.7 gm per day, as against an intake of 9 to 11 gm in untreated rats offered the same diet ad libitum.

Carotene and vitamin A. Three different aspects of vitamin A metabolism were studied: the efficiency of conversion of carotene to vitamin A, the fecal excretion of unchanged carotene after administration of the pro-vitamin, and the ability of the liver to store vitamin A. In all of these experiments no vitamin A supplement was given during the 4-week period of emetine treatment.

In the first series of experiments 18 emetine-treated and 12 control rats received during the last 6 days of the 4th week of the experiment a daily dose of 0.15 mg of crystalline β-carotene dissolved in 0.1 ml of olive oil. Twenty-four hours after the last orally-administered dose, the rats were killed and their livers examined for vitamin A. During the 6 consecutive days of the administration of β-carotene, the feces were collected for carotene determination and kept at 2°C.

The livers of the emetine-treated rats contained 54.1 ± 8.2 I.U., or 12.5 ± 1.7 I.U. per gram of liver. The figures for the controls were 49.4 ± 6.8 I.U. of vitamin A per liver or 12.3 ± 1.7 I.U. per gram of liver. It may be mentioned that the livers of the emetine-treated rats were found to be enlarged. In 82 treated rats an average liver weight of 4.4 gm or 6.6% of body weight was observed, as against 4.6 gm or 4.6% of body weight found in 91 control rats.

The total excretion of carotene amounted to 144 ± 26 μg or 16% of the dose for the treated rats, and 102 ± 12 μg, or 12% of the dose, for the controls. This difference was found to be statistically insignificant.

In the second series 6 treated and 6 untreated rats received 21,000 I.U. of vitamin A per day during the last three days of the 4th week of the experiment. Twenty-four hours later they were killed and the vitamin A content of their livers determined. In the livers of the treated rats 11,230 ± 1740 I.U. of vitamin A were found, or 2000 ± 282 I.U. per gram of liver. The corresponding figures for the control rats were 10,360 ± 1170 I.U. per liver and 1850 ± 233 I.U. per gram of liver.

These experiments show that emetine treatment does not affect the ability of the liver to store vitamin A after administration of either the pro-vitamin or the vitamin itself. Furthermore, absorption of carotene and its conversion to vitamin A appear to be unimpaired during such treatment.

Thiamine. Emetine-treated and control rats excreted similar amounts of thiamine in the urine (table 1). The amount of this vitamin found in the liver was, however, significantly lower in the treated rats than in the controls. In view of the fact that a specific biochemical rôle has so far only been demonstrated for the phosphorylated form of the vitamin, the percentage of free and of phosphorylated thiamine (cocarboxylase) was determined in liver and heart. The percentage of phosphorylated thiamine found in the livers of 26 treated rats was 93.3 ± 1.6, as against 95.3 ± 1.1% in the livers of 24 control rats. This small difference is not statisti-

cally significant. Since the total amount of thiamine in the livers of the treated rats was reduced, a corresponding significantly lower concentration of cocarboxylase was found also. The amount of cocarboxylase was 15.1 ± 1.07 µg per liver of treated rats, and 20.6 ± 1.45 µg in the livers of the controls.

No significant difference was found in the total amount and the percentage of phosphorylated thiamine in the heart.

TABLE 1

Urinary excretion and liver level of B vitamins
Means and standard errors

VITAMIN	EMETINE	URINE		LIVER		
		No. of rats [1]		No. of rats	Total	
			µg per day		*mg*	*µg per gm*
Thiamine	+	10	0.40 ± 0.13	26	16.1 ± 1.07	4.62 ± 0.43
Thiamine	—	10	0.43 ± 0.10	24	21.5 ± 1.51 [2]	4.89 ± 0.34
Riboflavin	+	10	11.9 ± 1.0	9	84.4 ± 9.5	19.5 ± 1.2
Riboflavin	—	10	11.0 ± 1.2	9	79.3 ± 4.9	22.0 ± 0.9
Nicotinic acid	+	12	34.1 ± 4.8 [3]	22	619 ± 31	118 ± 6.4
Nicotinic acid	—	14	31.8 ± 2.2 [3]	22	607 ± 28	128 ± 4.8
Pantothenic acid	+	10	52.0 ± 3.0	9	340 ± 38	73 ± 3.9
Pantothenic acid	—	10	46.1 ± 4.4	9	290 ± 37	80 ± 6.0
Biotin	+	10	72.1 ± 12.7 [4]	5	6.10 ± 1.4	1.04 ± 0.22
Biotin	—	10	56.2 ± 4.8 [4]	5	6.60 ± 1.1	1.52 ± 0.23
Folacin	+	12	1.20 ± 0.39	11	6.45 ± 0.92	1.50 ± 0.18
Folacin	—	12	0.95 ± 0.27	12	9.03 ± 1.23 [5]	2.11 ± 0.27 [6]
Citrovorum factor	+	6	45.0 ± 6.2 [4]			
Citrovorum factor	—	6	57.0 ± 34.0 [4]			

[1] Each rat underwent two urine collection periods.
[2] Difference significant at 1% level.
[3] The figures indicate N^1-methylnicotinamide.
[4] The figures indicate millimicrograms.
[5] Difference significant at 2% level.
[6] Difference significant at 5% level.

The hearts of 11 treated rats contained 1.65 ± 0.15 μg of thiamine per organ, or 5.57 ± 0.42 μg per gram of heart tissue. The corresponding figures for the controls were 1.92 ± 0.20 μg per organ and 5.21 ± 0.37 μg per gram of tissue. Practically all of this vitamin was phosphorylated.

Riboflavin, nicotinic acid, pantothenic acid and biotin. The urinary excretion of riboflavin, N-methyl-nicotinamide, pantothenic acid, and biotin in emetine-treated rats did not differ greatly from that of pair-fed controls (table 1). The liver levels of riboflavin, nicotinic and pantothenic acids and of biotin were similar in both groups. It is noteworthy, however, that emetine poisoning does not seem to impair the ability of the body to methylate nicotinamide.

Folacin. The excretion of folacin in urine was not found to be affected by the administration of emetine (table 1). The livers of the treated rats, however, contained significantly less folacin than those of the controls. Perhaps some folacin is stored elsewhere than in the liver.

Citrovorum factor. Sauberlich ('49) was the first to show that injections of folacin in rats or human beings considerably increased the urinary excretion of CF. These findings strongly suggested that CF is a derivative of folacin. Studies by Nichol and Welch ('50) and Welch, Nichol, Anker and Boehne ('51) have shown that ascorbic acid promotes the formation of CF from folacin. Thus it was of interest to us to study the ability of emetine-poisoned rats to convert folacin into CF.

The urine of 12 emetine-treated and of 12 control rats was collected during three days and examined for folacin and CF. Following this first collection period all rats were injected subcutaneously with 1.0 mg of folacin per 100 gm of body weight per day. Urine was again collected for three days and the percentage of the administered dose of folacin excreted, either as folacin or as CF, was determined. This second period was followed by a third period, during which all rats received in addition to 1.0 mg of folacin a daily subcutaneous injection of 10 mg of ascorbic acid, dissolved in normal saline, per 100 gm of body weight. The urine was

collected again during three days, and the amount of folacin and CF determined. The results obtained are shown in table 2.

It follows, from table 2, that emetine-treated rats excrete a significantly smaller percentage of an injected dose of folacin than do normal controls. Furthermore, ascorbic acid seems to stimulate the conversion of folacin into CF in both groups, but the poisoned rats, when injected with folacin together with ascorbic acid, excrete significantly less CF than the control animals.

TABLE 2

Urinary excretion of folacin and citrovorum factor after injection of folacin and ascorbic acid

The figures indicate the amounts of folacin and CF excreted, expressed as percentage of dose of folacin administered
Means and standard errors

GROUP	EXCRETION FOLLOWING ADMINISTRATION OF			
	Folacin		Folacin with ascorbic acid	
	Folacin	CF	Folacin	CF
Experimental	20 ± 2.2	0.38 ± 0.06	23 ± 2.5	0.77 ± 0.10
Control	35 ± 2.7	0.52 ± 0.11	32 ± 2.6	1.13 ± 0.14

These results, together with the lower storage of folacin in the livers of emetine-treated rats, suggest that the treated rats use or destroy more folacin than the untreated animals. The same relationship appears to hold good for CF.

Ascorbic acid. Vitamin C metabolism was studied in the following way: ascorbic acid concentration in liver and adrenals and its urinary excretion were examined in 11 treated and 11 untreated rats. In a second experiment, ascorbic acid synthesis was stimulated by feeding a diet containing 1.5 gm of sodium phenobarbital per kilogram of ration. Again the ascorbic acid content of the livers and adrenals as well as the urinary excretion was determined in these animals. The results are shown in table 3.

The results obtained indicate that emetine-treated rats excreted much less ascorbic acid in their urine than controls,

TABLE 3

Urinary excretion and level of ascorbic acid in adrenals and liver

Means and standard errors

NO. OF RATS	EMETINE	ASCORBIC ACID IN URINE	ADRENALS			ASCORBIC ACID IN LIVER	
			Weight		Ascorbic acid		
		mg per day	*mg*	*mg per 100 gm rat*	*µg per adrenals*	*mg per liver*	*mg per 100 gm liver*
			Time of treatment: 4 weeks				
11	+	0.03 ± 0.03 [1]	23 ± 1.1 [2]	36.4 ± 3.2 [2]	121 ± 7.9 [2]	1.02 ± 0.11 [1]	26.7 ± 2.5 [1]
11	—	0.22 ± 0.07	16 ± 0.8	16.4 ± 1.0	74 ± 7.3	1.43 ± 0.06	34.7 ± 1.9
6 [3]	—		18 ± 0.8	16.3 ± 2.0	63 ± 8.3	1.58 ± 0.07	30.2 ± 1.8
9 [4]	+	2.66 ± 0.42	23 ± 1.7 [2]	24.4 ± 1.8 [2]	117 ± 12.0 [2]	1.51 ± 0.12 [2]	28.2 ± 2.6 [2]
12 [4]	—	3.43 ± 0.32	17 ± 0.5	13.8 ± 0.6	62 ± 4.2	2.37 ± 0.09	41.4 ± 1.7
			Time of treatment: three weeks				
6	+		18 ± 1.0 [2]	31.4 ± 3.7 [5]	72 ± 5.4 [5]	0.76 ± 0.03	21.6 ± 2.4
6	—		13 ± 1.3	19.2 ± 1.4	50 ± 4.9	0.66 ± 0.06	25.6 ± 2.2
6 [3]	—		14 ± 0.9	16.0 ± 0.9	46 ± 3.5	1.47 ± 0.05	31.2 ± 1.7
			Time of treatment: two weeks				
6	+		14 ± 0.8 [5]	21.6 ± 0.9 [2]	58 ± 4.7 [6]	0.90 ± 0.08	30.4 ± 0.9
6	—		11 ± 0.4	15.7 ± 0.9	43 ± 3.9	0.98 ± 0.09	29.5 ± 1.1
			Time of treatment: one week				
6	+		12 ± 1.5	27.8 ± 1.1 [6]	51 ± 0.1 [6]	0.59 ± 0.04 [2]	27.5 ± 1.4 [2]
6	—		14 ± 0.8	24.5 ± 1.0	44 ± 3.4	1.40 ± 0.09	38.4 ± 1.2

[1] Difference between experimental and pair-fed control groups significant at the 2% level.
[2] Difference between experimental and pair-fed control groups significant at the 0.1% level.
[3] Fed ad libitum.
[4] Rats treated with sodium phenobarbital.
[5] Difference between experimental and pair-fed control groups significant at the 1% level.
[6] Difference between experimental and pair-fed control groups significant at the 5% level.

the difference, however, being significant only in those animals which had not been given barbiturate. Barbiturate stimulated excretion, and probably also synthesis, of ascorbic acid in both groups to an almost equal extent. The adrenals of the emetine-treated rats were enlarged and weighed significantly more than those of the control animals. This hypertrophy probably results from the stress imposed upon the animals following repeated administration of the drug. The amount of ascorbic acid in the adrenals of the emetine-treated rats exceeded by far that found in the adrenals of the controls; the livers of the treated rats, however, contained much less ascorbic acid than those of the control animals.

This increase in ascorbic acid content in adrenals that were hypertrophied and thus obviously subjected to stress seemed to be surprising, since stress frequently leads to an ascorbic acid depletion of this organ (Long, '47). We therefore studied the concentration of ascorbic acid in the adrenals and livers of: (1) rats receiving shorter periods of emetine treatment (one to three weeks), and (2) normal rats fed ad libitum, since the restriction of food intake imposed upon the pair-fed controls may possibly in itself constitute a stress situation. From table 3 it may be observed that even after only one week of emetine treatment, the adrenals of the treated animals weighed more and contained a higher ascorbic acid concentration than those of the control animals. On the other hand, the ascorbic acid content of the liver was found to be reduced, although to a lesser degree, even after only one week of emetine administration. The weight of the adrenals of the pair-fed rats, as well as their ascorbic acid content, did not differ significantly from the corresponding values obtained on animals fed ad libitum.

It would seem, therefore, that the food restriction as employed in our experiments does not constitute a stress situation leading to a visible adrenal hypertrophy.

From the results of our experiments the following has been demonstrated:

1. Prolonged treatment with emetine leads, like other agents causing a non-specific stress, to a marked hypertrophy of the adrenal glands.

2. The ascorbic acid content of the adrenals is increased, and that of the liver decreased in emetine-treated rats.

3. The urinary excretion of ascorbic acid is reduced in rats poisoned by emetine.

4. Synthesis of ascorbic acid, following stimulation with barbiturate, is not impaired by treatment with emetine.

DISCUSSION

In our experiments we have been able to show that prolonged treatment with emetine produces certain changes in the metabolism of some vitamins, whereas that of others does not seem to be affected. No deviation from the normal metabolism was found for vitamin A, riboflavin, nicotinic and pantothenic acids, and biotin. Slight deviations, however, were found in the metabolism of thiamine and folacin, the amounts of these latter vitamins in the liver decreasing during prolonged emetine treatment. Moreover, emetine-poisoned rats excrete a smaller percentage of an injected dose of folacin, and less CF after stimulation of its synthesis by ascorbic acid than do normal controls. It appears that the treated rats use or destroy more folacin and CF than the untreated animals. The possibility, however, of an inefficient conversion of folacin into CF in emetine-poisoned rats cannot be dismissed.

It is not surprising that prolonged emetine treatment induces the anatomical changes typical of stress in the adrenals. It is, however, noteworthy that the hypertrophied adrenals contain more ascorbic acid than normals. This finding supports the view expressed by Pinchot, Close and Long ('49) that the concentration of ascorbic acid alone is not a reliable index of the functional state of the adrenal gland which has been subjected to a prolonged stress. Similarly, Sayers ('50) points out that adrenal ascorbic acid may be regarded as a reliable index of adrenal cortical activity in acute experi-

ments on healthy rats only, whereas in experiments of longer duration, involving a prolonged stress, factors other than the rate of secretory activity of the adrenal cortex play an important or even dominant rôle in determining the ascorbic acid level in the gland. The increased concentration of this vitamin in the adrenals observed in our experiments suggests that the synthesis of ascorbic acid is not impaired in emetine poisoning. It may perhaps be understood as the result of the increased synthesis of this vitamin which compensates for an increased requirement. Similarly, Dugal and Thérien ('49) have shown that prolonged exposure of guinea pigs and rabbits to cold also leads to an increase of ascorbic acid in the adrenals. As a matter of fact, our emetine-treated rats responded to stimulation of synthesis by barbiturate as well as normal rats.

It is interesting to compare our results with those obtained by Shils, Sass, Wolke, Marks, Goldwater and Berg ('51). These authors studied the livers of rats subjected to treatment with carbon tetrachloride or with benzene. The livers of the first group of rats showed a diminished concentration of thiamine, riboflavin, nicotinic acid and vitamin A, whereas treatment with benzene was without any effect on the liver level of these vitamins.

SUMMARY

1. Young growing rats were treated repeatedly with a small daily dose of emetine.

2. Such emetine treatment caused a reduction in food intake, resulting in a diminished growth rate.

3. No change from the normal was found in the metabolism of vitamin A, riboflavin, nicotinic and pantothenic acids, and biotin. Phosphorylation of thiamine and methylation of nicotinic acid were also unimpaired.

4. Emetine-treated rats stored smaller amounts of thiamine and folacin in their livers than pair-fed untreated controls.

5. Emetine-treated rats excreted a smaller percentage of folacin in their urine, after injection of a test dose of this

vitamin. Likewise, the urinary excretion of citrovorum factor after injection of folacin together with ascorbic acid, was also decreased in emetine-treated rats, in comparison with controls.

6. Emetine-treated rats excreted less ascorbic acid in their urine; their adrenals contained more and their livers less ascorbic acid than the corresponding organs of untreated controls. The synthesis of ascorbic acid following stimulation with barbiturate was not impaired in emetine-treated animals.

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THE USE OF DEPLETED RATS FOR INVESTIGATIONS OF VITAMIN B_{12} AND UNIDENTIFIED FACTORS

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Before the isolation of vitamin B_{12} several investigators, Spitzer and Phillips ('46), Zucker et al. ('44 and '48) and Cary, Hartman, Dryden and Likely ('46) obtained inferior reproduction in rats or abnormal growth of weanling rats when the breeding females were fed all-vegetable or purified rations. Results were improved by such materials as liver powder, fish meal, meat, crude casein, milk products, egg yolk, alfalfa and other leafy materials. Following the isolation of vitamin B_{12} the outstanding responses of depleted rats to vitamin B_{12} reported by Hartman et al. ('49), Schultze ('49), Emerson ('49) and Cuthbertson and Thornton ('52) and others have made it appear possible that vitamin B_{12} was responsible for the main if not the entire activity of the "animal protein factor," "zoopherin" or "factor X." The ability of vitamin B_{12} to counteract thyrotoxicity in rats fed iodinated casein has been used as a basis for vitamin B_{12} assays by Frost, Fricke and Spruth ('49) and Tappan, Lewis, Register and Elvehjem ('50); however, the extent to which unidentified factors may affect the response of hyperthyroid rats to B_{12} has apparently not been investigated. The purpose of this paper is to present the results of experiments which were conducted in conjunction with assays of various sources of

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vitamin B_{12} and unidentified factors using weanling rats which were depleted of vitamin B_{12} and unidentified factors by restricting the breeding ration in these factors.

TABLE 1

Composition of diets

CONSTITUENTS	BREEDING RATION	GROWING RATION
	%	%
Yellow corn meal	16.20	33.25
Molasses	3.00	
Dehydrated alfalfa	4.00	
Solvent soybean oil meal	20.50	19.85
Corn gluten meal		16.00
Cottonseed meal		18.20
Brewers yeast	3.00	7.00
Linseed meal	7.00	
Ground wheat	30.00	
Wheat germ meal	12.00	
Salt	.23	.23
Trace minerals [1]	.02	.02
$Ca_3(PO_4)_2$ (Bone meal)	2.70	3.50
Vitamin A supplement (10,000 I.U./gm)	.15	.15
Vitamin D_2 supplement (440 U.S.P. U./gm)	1.20	1.00
Choline dry mix (25%)		.80
	100%	100%

[1] Trace minerals were 98.5% manganese sulphate, 1.0% sodium thiosulphate and 0.5% potassium iodide.

EXPERIMENTAL

Wistar strain weanling rats weighing 40 to 50 gm were used for the growth tests. The breeding females which produced the young for these studies were raised to maturity on a standard rat breeder diet [2] and were fed the vegetable-protein breeder ration during gestation and lactation. The composition of this ration is given in table 1. Successive litters were obtained by giving the females a two-week resting period on the standard breeder diet between litters and then repeating the use of the vegetable-protein breeding ration during gestation and lactation. Uniform sex and litter dis-

[2] Purina Laboratory Chow.

tribution of young on the rations was practiced. Ten males and 10 females were used in the negative control and 5 males and 5 females in other groups. The composition of the growing ration fed to the weanling young is given in table 1. It was a vegetable-protein ration and contained 30% protein with approximately equal amounts of protein supplied by soybean oil meal, cottonseed meal and corn (yellow corn meal and corn gluten meal). Ad libitum feeding was used throughout.

The growth tests were of 4-weeks duration, and initial as well as 28-day individual weights were recorded. High mortality was characteristic of the negative control. Dead weights of animals which died after one week on test were included in the average weights.

RESULTS

The growth responses of depleted rats to varying levels of vitamin B_{12}, vitamin B_{12a} and pseudovitamin B_{12} as well as to 3% fish solubles are given in table 2, experiment 1. Increased weight gains were observed with increasing levels of vitamin B_{12} up to a level of 13.75 μg of vitamin B_{12}/kg of diet. Vitamin B_{12a} gave approximately the same growth as vitamin B_{12} when fed at a comparable level. Pseudovitamin B_{12} was entirely inactive with the depleted rats. The growth obtained with pseudovitamin B_{12} was inferior to that of the negative control group which would indicate the possibility that pseudovitamin B_{12} was aggravating the deficiency of vitamin B_{12} and could be considered indicative that pseudovitamin B_{12} had anti-vitamin B_{12} activity. The growth response to 3% fish solubles was approximately double that with optimum levels of vitamin B_{12} and is indicative of the magnitude of the response to the unidentified factors present in the fish solubles.

The growth responses of depleted rats to various natural products containing unidentified factors or vitamin B_{12} are given in table 2, experiment 2. Dried whole liver and liver extract gave excellent growth responses equal to the response

TABLE 2

Average 28-day gains of depleted rats receiving various forms of vitamin B_{12}, antibiotics or sources of unidentified growth factors

SUPPLEMENT TO BASAL	AVERAGE 4-WEEK GAIN
	gm
Experiment no. 1	
None	38.0
1.1 μg Vitamin B_{12}/kg [1]	39.5
3.3 μg Vitamin B_{12}/kg	55.7
6.85 μg Vitamin B_{12}/kg	66.7
13.75 μg Vitamin B_{12}/kg	72.3
27.5 μg Vitamin B_{12}/kg	74.0
3.0% Fish solubles	103.0
6.85 μg Vitamin B_{12a}/kg [2]	76.0
6.85 μg Pseudovitamin B_{12}/kg [2]	21.0
Experiment no. 2	
None	27.5
13.75 μg Vitamin B_{12}/kg	47.6
27.5 μg Vitamin B_{12}/kg	50.0
13.75 μg Vitamin B_{12}/kg + 5% whole liver, dried [3]	108.0
13.75 μg Vitamin B_{12}/kg + 2% liver extract [4]	115.0
13.75 μg Vitamin B_{12}/kg + 5% liver residue [4]	46.0
4% Dried whey	25.0
13.75 μg Vitamin B_{12}/kg + 4% dried whey	106.0
13.75 μg Vitamin B_{12}/kg + 5% butyl fermentation solubles	74.0
4% Fish meal	66.0
4.7% Fish solubles	113.0
Experiment no. 3	
None	42
13.75 μg Vitamin B_{12}/kg	67
13.75 μg Vitamin B_{12}/kg + 0.0015% streptomycin [5]	68
13.75 μg Vitamin B_{12}/kg + 0.0015% bacitracin [6]	72
13.75 μg Vitamin B_{12}/kg + 0.0015% aureomycin [7]	75
13.75 μg Vitamin B_{12}/kg + 0.0015% procaine penicillin [8]	76
13.75 μg Vitamin B_{12}/kg + 0.0015% terramycin [5]	76
13.75 μg Vitamin B_{12}/kg + 0.005% 3-nitro, 4-hydroxyphenyl-arsonic acid [9]	69
3% Fish solubles	107
Experiment no. 4	
None	32
13.75 μg Vitamin B_{12}/kg	72
13.75 μg Vitamin B_{12}/kg + 5% malt sprouts [10]	73
13.75 μg Vitamin B_{12}/kg + 5% grass juice factor [11]	78
13.75 μg Vitamin B_{12}/kg + 4% mucin [12]	70
13.75 μg Vitamin B_{12}/kg + 15% mg lyxoflavin/kg [13]	69
13.75 μg Vitamin B_{12}/kg + 55 μg orotic acid/kg [14]	80
3% Fish solubles	118
13.75 μg Vitamin B_{12}/kg + vitamin supplement [15]	72

[1] Crystalline vitamin B_{12} — Merck and Co., Inc.
[2] Crystalline vitamin B_{12a} and pseudovitamin B_{12} — Pacific Yeast Products, Inc.
[3] Whole liver, dried — Viobin Laboratories.
[4] Liver extract and liver residue — Wilson Laboratories.
[5] Streptomycin and terramycin — Chas. Pfizer and Co.
[6] Bacitracin — Commercial Solvents Corp.
[7] Aureomycin — Lederle Laboratories.
[8] Procaine penicillin — Merck and Co.
[9] 3-nitro, 4-hydroxyphenylarsonic acid — Specifide, Inc.
[10] Malt sprouts — Pabst Brewing Co.
[11] Grass juice factor — Cerophyl Laboratories.
[12] Mucin — Wilson Laboratories.
[13] Lyxoflavin — Merck and Co.
[14] Orotic acid — Dougherty Chemicals.
[15] Vitamins added in mg/kg: thiamine, 4; riboflavin, 6; pyridoxine, 5; niacin, 4; pantothenic acid, 4; choline, 2000; inositol, 200; menadione, 2; biotin, 0.2; folic acid, 0.2 and alpha-tocopherol acetate, 200.

obtained with fish solubles. The 4.74% level of fish solubles used in this experiment supplied 13.75 μg of vitamin B_{12}/kg based on microbiological assay using a pad plate modification of the U.S.P. method with *L leichmannii* ATCC no. 7833. There was no growth response when the liver residue was added to the basal diet in the presence of vitamin B_{12}. Dried whey in the absence of vitamin B_{12} did not give a growth response. However, in the presence of added vitamin B_{12} a very good response to whey was obtained. Butyl fermentation solubles with added vitamin B_{12} gave a response which was approximately half that obtained with fish solubles. A response of about the same magnitude was obtained with fish meal in the absence of vitamin B_{12}. It is possible that the level of vitamin B_{12} may have limited growth of the group fed fish meal.

In view of the growth responses obtained with antibiotics and various arsonic acid compounds in other species it seemed desirable to test these materials in pure form with depleted rats. Results from these experiments are given in table 2, experiment 3 and show that none of the antibiotics or 3-nitro, 4-hydroxyphenylarsonic acid were active in stimulating growth of depleted rats.

The results with various products which have been considered as possible sources of unidentified factors are given in table 2, experiment 4. Malt sprouts and lyxoflavin were without unidentified factor activity when added to vitamin B_{12} under the conditions of this experiment. There was indication of some activity when orotic acid was added at the level of 55 μg/kg. Further work is in progress to determine if orotic acid at a higher level may give a greater growth response. Grass juice factor with added vitamin B_{12} produced only slightly better gains than vitamin B_{12} alone.

One group of rats received a B vitamin mixture in order to determine if 7% brewers yeast in the growing ration furnished an adequate level of B vitamins or if the growth responses to sources of unidentfied factors were due to increased

levels of known B vitamins. No detectable response to the vitamin mixture was observed.

DISCUSSION

The results of this investigation clearly demonstrate the requirement of rats for unidentified factors in addition to vitamin B_{12}. The growth of depleted rats which received fish solubles, dried whey, liver extract or whole liver in addition to vitamin B_{12} was at least double that with vitamin B_{12} alone. A smaller response was obtained with butyl fermentation solubles and fish meal.

There was a fairly good correlation between the potencies of many biological materials for rats and published data on distribution of certain unidentified factors for poultry. A chick growth response to whey has been reported by Berry, Carrick, Roberts and Hauge ('43) and by Hill, Scott, Norris and Heuser ('44). Menge, Combs, Hsu and Shorb ('52) obtained evidence that two distinct factors for chicks were present in liver preparations. Fish meal and fish solubles have been shown to be good sources of unidentified growth factors for chicks by Lillie, Sizemore and Bird ('53) and others.

There was only a small response of the depleted rats to grass juice factor. Kohler and Graham ('51) obtained a growth response in chicks to grass juice factor and Hansen, Scott, Larson, Nelson and Krichevsky ('53) reported a chick growth response to dehydrated or sun-cured alfalfa as well as to grass juice concentrate. There also is evidence of an unidentified growth factor for chicks in brewers yeast as reported by Schumacher, Heuser and Norris ('40), Kohler and Graham ('51) and others. The low magnitude of the rat response to grass juice factor may be explained on the basis that the rat breeding ration used in the present studies contained dehydrated alfalfa and both the breeding and growing rations contained brewers yeast. The rat results are supporting evidence to the view that the factor present in grass juice concentrate, alfalfa and yeast is distinct from the fish and whey factors. Lyxoflavin, shown by Cooperman, Marusich,

Scheiner, Drekter, De Ritter and Rubin ('53) to be active for rats on a riboflavin-low ration gave no response with the depleted rats used in the present study, probably because of an adequate riboflavin level in the rations supplied by brewers yeast. Mucin, shown by Gyorgy, Mello, Torres and Barness ('53) to produce a growth response in rats, was inactive under the conditions of the present experiments which would indicate that either similar mucin constituents were present in the ingredients of the basal diet or that the depleted rats were unable to respond to mucin because of a primary deficiency of the fish or whey factor. Orotic acid has been isolated from whey and distiller's solubles and has been shown to produce a growth response in depleted rats at a level of 1 mg/100 gm of ration by Manna and Hauge ('53). In the present investigation only a small response to orotic acid was obtained which was considered not significant. However, the level of feeding was only 5.5 μg/100 gm, and in view of the higher level of orotic acid fed by Manna, further work with orotic acid at higher levels is being carried out.

There is no explanation for one apparent difference in the response of depleted rats and reported results with hyperthyroid rats with respect to liver residue. Ershoff ('50) obtained no response in hyperthroid rats to vitamin B_{12} and a positive response to liver residue; however, in the present studies with depleted rats the reverse was true.

The results of these investigations indicate certain advantages and disadvantages in the technique used for running assays of vitamin B_{12} and unidentified factors. As with chick assays, the rat assay using depleted animals is not specific for vitamin B_{12}, since both species respond to unidentified factors which occur with vitamin B_{12} in most biological materials. It is possible that the assay may be made more specific for vitamin B_{12} by the inclusion of dried whey, or other sources of unidentified factors low in or devoid of vitamin B_{12}, in the growing ration or in the breeding ration. These possibilities are being investigated at the present time. The data indicate that the depleted rat responded well to the same

unidentified factors as are present in fish, liver, dried whey and distillers solubles which are also active for chicks. Moreover, the rat assay would appear to have certain advantages over the chick assay for isolation and fractionation work on unidentified factors because of the smaller quantity of sample required for assay and because of the greater magnitude of the response with the depleted rat than has been obtained by most investigators using depleted chicks.

SUMMARY

Depleted weanling rats from breeder females which received a vegetable-protein ration during gestation and lactation were used for investigations of vitamin B_{12} and unidentified factors.

The weanling rats responded to vitamin B_{12a} and to graded levels of vitamin B_{12} but not to pseudovitamin B_{12}. The addition of unidentified factors produced more than twice as much gain as was observed with 13.75 μg of vitamin B_{12}/kg.

With vitamin B_{12} in the ration, dried whole liver, dried liver extract, fish solubles and dried whey produced greater gains than fish meal and fermentation solubles. Antibiotics, 3-nitro, 4-hydroxyphenylarsonic acid, malt sprouts, liver residue, lyxoflavin and mucin were inactive as sources of unidentified factors. The response with grass juice factor and orotic acid were small and of doubtful significance.

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Calif., for crystalline vitamin B_{12a} and pseudovitamin B_{12}; to Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for aureomycin; and to Wilson Laboratories, Chicago, Ill., for mucin and to Pabst Brewing Co., Milwaukee, Wis., for malt sprouts.

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NUTRITIONAL STATUS OF THE AGING

I. HEMOGLOBIN LEVELS, PACKED CELL VOLUMES AND SEDIMENTATION RATES OF 577 NORMAL MEN AND WOMEN OVER 50 YEARS OF AGE[1]

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NINE FIGURES

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This study was a part of the western regional cooperative project on nutritional status of selected population groups. The California phase of the research was carried out in 1948–1949 on 280 men and 297 women over 50 years of age, residing in San Mateo County, California. This area was chosen because of its well organized department of public health and welfare, because its people were thought to be relatively stable as to residence, and because of its proximity to the home laboratory. The object of the study was to determine the food habits of these aging people, to learn whether these habits could be connected in any way with their physical condition, blood composition, density of bone, and other measurable characteristics. The number of subjects reported upon for various aspects of the study will vary somewhat since

[1] California Agricultural Experiment Station, Berkeley. This study was part of the Western Regional Research Project W 4 on nutritional status of population groups in California. It was financed in part from 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.

all determinations were not made or were not accepted for each of the 577, the maximum number examined.

The subjects were obtained by appeal for volunteers, particularly to organized groups such as churches, clubs, labor unions and community service organizations.[2] No effort was made to secure a representative sample of the population since the research was directed toward correlations more than toward establishment of the nutritional status of any population segment. Only apparently healthy peoeple were accepted for study, the criterion being that the volunteer consider himself well and declare that he had not consulted a physician for three months preceding the examination.

The first group examined at the Crystal Springs county home was an exception to the rest in that, except for three who were 50 to 59 years old, these were all men over 60 years of age, who had for the most part lived for some years in the home and had therefore been exposed to a uniform food supply. More reliable than usual food intake records were secured for these 47 men since the nutritionists were able to make three-day diet records by direct observation.

The other subjects were living in their own homes. The economic status of these people offered a wide range from those living on old age pensions to those in the highest tax brackets. The large majority, 80% according to the interviewers' estimates, were in the comfortably secure but not wealthy middle class. No attempt was made to secure exact data on income but the interviewers[3] were able to make a rough estimate from the living conditions which they observed.

PROCEDURE

A field laboratory trailer was utilized for taking the x-rays, drawing the blood samples by venipuncture and for the

[2] The effective assistance of Amelia Feary in enlisting the volunteer participants is acknowledged.

[3] The interviews and collection of the dietary records and histories were accomplished by Clara Beth Young, Madalyn R. Tomassetti, Jane Baldwin and Helen W. Hubbard.

hemoglobin, hematocrit, serum protein, glucose and ascorbic acid determinations which were performed immediately after the blood was secured. The vitamin A, carotene, cholesterol, nonprotein nitrogen, uric acid and creatinine determinations were made later in the Berkeley laboratory. All blood and serum samples for the latter tests were preserved in the frozen condition from the time they were secured until the analyses were undertaken. The blood was divided in the trailer into portions oxalated with ammonium and potassium oxalates for hemoglobin, hematocrit, sedimentation rate and glucose tests, with lithium oxalate for the nonprotein nitrogen, uric acid and creatinine, and unoxalated blood which was centrifuged after standing 15 to 20 minutes to obtain serum for the other determinations. The qualitative urine tests were made at once in the trailer laboratory.

Diet records

The dietary data were obtained from 7-day food intake records that were kept by the participants who lived in their own homes, and from three-day records obtained by the nutritionists for those living in the Crystal Springs Home. Household measures were used. A nutritionist visited each person in his own home to give instructions for keeping the records and to answer questions concerning the forms to be used. Envelopes were supplied for mailing each day's record to the office as soon as it was completed. When the record was received, it was examined by the nutritionist who visited or telephoned to the person as soon as possible if anything questionable was found in the record.

At the end of the 7-day period the nutritionist took a dietary history by the interview method, using a modification of the check list approved for use in the western regional cooperative nutrition project. This dietary history has been used to determine whether the 7-day record represented the customary intake of the subject with respect to various types of food, but it was not used in the calculation of the nutrient value of the diet.

Calculation of the nutrient value of the 7-day food intake was done by the use of IBM food value master cards supplied by the Bureau of Human Nutrition and Home Economics, and an additional lot of approximately 200 cards which were made by us from values obtained from various sources (Watt and Merrill, '50; U. S. Public Health Service, '47; Bowes and Church, '46). The three-day records of the men at the Crystal Springs Home were calculated from the values of foods prepared and served under the observation of the nutritionists. The fat values were calculated from the calories that remained after deducting the caloric value of the protein, carbohydrate and alcohol contained in beverages and food. The cholesterol values were calculated from the tables of Okey ('45) and additional values supplied by her. In certain cases of proprietary foods the nutrient values were supplied by the manufacturer.

Vitamin and mineral supplements reported by the subjects were recorded but were not included in the main calculations of nutrient intake. Certain comparisons were made however, including these supplements, particularly in the attempted correlations of circulating and dietary vitamin concentrations.

Analytical methods

Hemoglobin. Hemoglobin was determined as alkaline hematin with a Leitz photoelectric colorimeter. This instrument had been calibrated for hemoglobin readings by determination of the oxygen capacity of venous blood by the Peters and Van Slyke ('32) procedure and comparison of the results with those obtained by the Leitz colorimeter. Blood oxalated with a mixture of ammonium and potassium oxalate was drawn to the 0.025 ml mark on a standard Leitz pipette, diluted with 0.1% sodium carbonate to 5.025 ml, gently rotated and expelled into the standard Leitz absorption tube and read within 5 minutes in the Leitz colorimeter.

Hematocrit. The method of Wintrobe ('46) was used to determine the volume of packed cells.

Sedimentation rate. The method of Wintrobe and Landsberg ('35) was followed, with oxalate as the anticoagulant. Readings were made after the tubes had stood one hour in a vertical position at 22° to 27°C. The tubes were then centrifuged 20 minutes or more at 3000 r.p.m. until packing of erythrocytes was complete and the cell volume read. The sedimentation rate was corrected when necessary for low cell volume.

Blood cell examination. A drop of blood from the needle was placed on each of two slides, films made and allowed to dry in air, then stained with Wright's stain, buffered to pH 6.4 and left until the stain acquired a metallic sheen. The smears were then rinsed, dried in air and examined under oil immersion for platelets and morphology of erythrocytes and leucocytes. The approximate number of leucocytes was noted but a differential count was not made.

Other determinations were made for serum protein, ascorbic acid, vitamin A, carotene, free and combined cholesterol, blood glucose, non-protein nitrogen, uric acid and creatinine. Reports on these analyses and on other aspects of the study will be made separately.

RESULTS

Hemoglobin. As shown in table 1 and figure 1 the hemoglobin values for the 235 men living in their own homes were at all ages higher than for the 296 women similarly grouped. The differences were significant except for ages 65 to 69 and over 75 years. The mean value for the women from 50 to more than 80 years of age was 13.4 gm per 100 ml of blood with no significant relationship to age except for a small drop after 80 years. The mean value for the men was 14.5 gm %, again with little variation except for a dip at 65 to 69 years and a compensating rise at 70 to 74 years as well as a drop thereafter. Whether there is a physiological reason for this variation cannot be established. These means with their standard errors and ranges are shown in figure 1.

If anemia is defined as that condition existing in persons having 11 gm % or less of hemoglobin, only a small number

of the subjects exhibited the condition. In the 50- to 59-year age group there were 1.04 cases per hundred examined, in the 60- to 69-year group 0.98, in the 70- to 79-year group 3.15 and over 80 years 4.16. Of the 235 men living in their own homes less than 3% had hemoglobin levels below 12 gm

TABLE 1

Hemoglobin and volume of packed cells (hematocrit) of 531 men and women over 50 years of age

AGE GROUP IN YEARS, SEX	NO. OF SUBJECTS	HEMOGLOBIN			VOLUME OF PACKED CELLS			MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION
		Mean	Range	Standard error	Mean	Range	Standard error	
		gm/100 ml blood	*gm/100 ml blood*		*% packed cells*	*% packed cells*		*%*
50–54								
Men	45	14.6	12.2–15.6	0.2	46.9	40–51	0.4	31
Women	49	13.3	8.4–17.0	0.2	44.4	38–53	0.4	30
55–59								
Men	41	14.7	13.0–18.3	0.2	47.9	42–63	0.6	31
Women	55	13.3	10.7–17.0	0.2	44.0	39–50	0.4	30
60–64								
Men	36	14.6	13.0–16.5	0.1	48.1	44–56	0.4	30
Women	57	13.5	11.4–16.5	0.2	44.8	39–51	0.3	30
65–69								
Men	39	14.0	10.4–17.0	0.2	46.0	35–56	0.6	31
Women	57	13.6	10.0–17.0	0.2	45.1	35–61	0.4	30
70–74								
Men	37	15.0	12.2–18.3	0.2	48.0	40–54	0.5	31
Women	40	13.5	10.4–17.0	0.2	44.9	40–55	0.5	30
75–79								
Men	19	14.1	9.7–17.0	0.4	46.8	35–55	1.1	30
Women	25	13.8	10.0–20.0	0.4	45.1	38–49	0.4	31
80 and over								
Men	18	13.4	9.0–16.0	0.6	44.7	33–52	1.0	30
Women	13	12.9	10.0–14.6	0.3	43.2	32–48	1.1	30
Total								
Men	235	14.5	9.0–18.3	0.3	47.1	33–63	0.7	30.6
Women	296	13.4	8.4–20.0	0.3	44.6	32–61	0.5	30.0

per 100 ml blood, and of the 296 women about 6% had similarly low levels. The incidence of these lower levels of hemoglobin increased in men over 65 years of age but was not affected by age in the women. The distribution of the hemoglobin levels is shown in figure 2.

The hemoglobin values found in this study are lower than those quoted by Wintrobe ('46) and Osgood ('35) for normal young adults. The mean values for males given by these

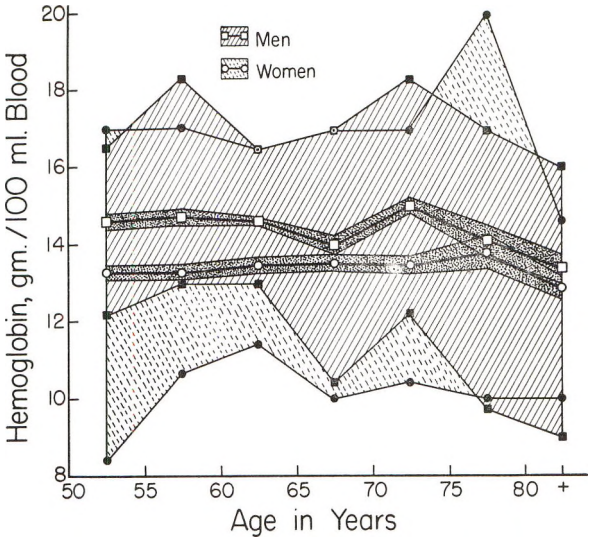

Fig. 1 Means, ranges and standard errors of hemoglobin of blood of 235 men and 296 women over 50 years of age.

authors are 16 and 15.8, for females 14 and 13.9. It is obvious that the difference between mean levels of the young and aging men, 1.3 or 1.5 gm per 100 ml blood, is considerably greater than that shown between those of young and aging women, 0.5 or 0.6 gm. The lessened blood loss which may follow the cessation of menstruation in the older women might compensate to some extent for any decreased blood production.

Earlier surveys of hemoglobin levels in aging subjects have generally covered smaller groups than those here reported.

Miller ('39) found 14.3 gm % in 160 men 60 to 104 years old. This is very close to the mean of our group. Williamson ('16) reported 15.82 in 81 men 61 to more than 76 years old and 15.39 in 71 women in this age group. Shapleigh, Mayes and Moore ('52) found 14.1 and 13.7 in 50 men and 50 women over 60 years of age. Newman and Gitlow ('43) with a similar group of subjects 65 to 104 years old reported 12.05 and 11.7. Fowler, Stephens and Stump ('41) found 13.1 gm % in 73 men aged 65 to 80 and 12.5 in 27 women of the same age range.

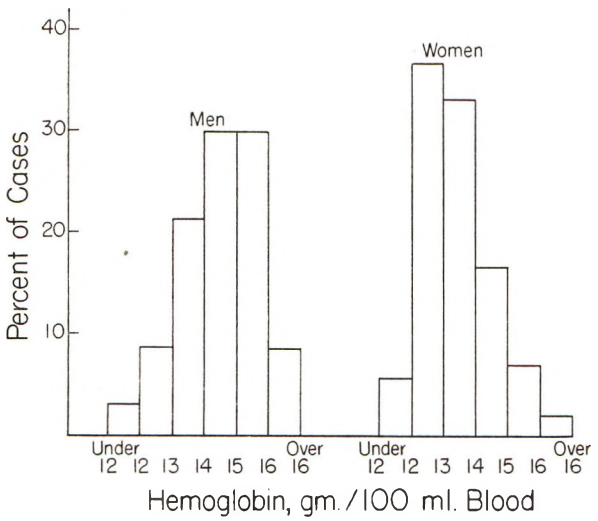

Fig. 2 Distribution of hemoglobin concentrations in 236 men and 296 women over 50 years of age.

Jefferson, Hawkins and Blanchaer ('53) found an average of 14.0 gm % hemoglobin in the blood of 330 men and 13.6 in that of 175 women, 50 to 97 years old. Capillary blood was used. Borsook et al. ('43) found in a study of 1170 men, mostly under 30 years of age, that 52% had less than 14.5 gm per 100 ml blood, and less than 5% equalled Wintrobe's normal of 16.0. This latter range corresponds fairly well with those of our series.

The analytical methods used in the studies quoted varied greatly in accuracy. With the exception of the Williamson

('16) study however, the mean values reported agree fairly well with those found in this investigation and indicate a definite trend toward lower hemoglobin levels in aging people as compared with those of healthy young adults.

Volume of packed cells. The mean volume of packed cells (table 1 and fig. 3) was larger in men than women at nearly all ages. Between 65 and 69 years and after 74 years there was no significant difference due to sex. The mean corpuscular hemoglobin concentrations were calculated and were found to be uniformly 31 and 30% for men and women.

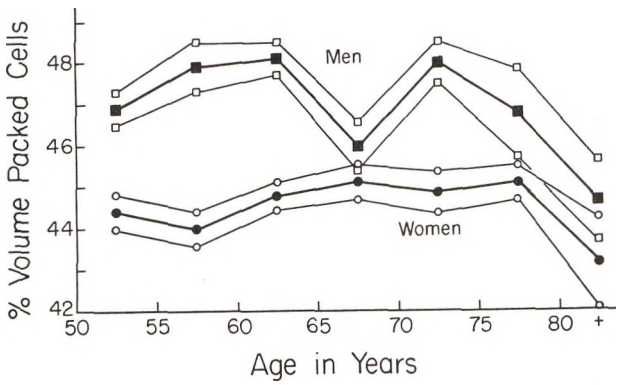

Fig. 3 Mean values and standard errors of volume of packed cells in blood of men and women over 50 years of age.

The volume of packed cells as measured in this study was equal to or larger than the values quoted by Wintrobe ('46) and Osgood ('35) for normal young adults, namely 47 and 44.8 for men, 42 and 41.8 for women. Our values for all subjects over 50 years of age were 47.1 for men and 44.6 for women. Shapleigh, Mayes and Moore ('52) found 42.1 for men and 40.8 for women, Fowler, Stephens and Stump ('41), 41.7 and 40.4, Newman and Gitlow ('43), 41.2 and 36.7, Olbrich ('48), in 41 men and 47 women over 60 years old, 45.1 and 42.0. Borsook et al. ('43) found a median of 47.9 in 1170 young men although the median hemoglobin was lower than that quoted by Wintrobe ('46).

The mean corpuscular hemoglobin concentration reported by Shapleigh, Mayes and Moore ('52) was 33.5% for both men and women as against 35 for young adults found by Wintrobe. Our values, 31 and 30, reflect the larger packed cell volumes found in our subjects (table 1 and figs. 1 and 3). Since no red blood cell counts were made in our study it is difficult to explain these high hematocrit values. Possibly the mean cell volumes were larger than those reported for other elderly subjects. Olbrich ('47) and Millet and Balle-Helaers ('32) described such an increase in mean cell diameter in their subjects over 60 and from 57 to 97 years of age.

Sedimentation rate. The sedimentation rates of the women for every age group were significantly higher than those of the men except at ages 60–64 and 80 years and over (table 2 and fig. 4). An upward trend with age was evident in the rates of the women but this was less marked in the men until after the age of 74.

The significance of the sedimentation rate is not always clear. It is known to be increased in infection and to be slightly greater in normal women than in normal men. It is not clearly related to any other constituent or constant of the blood except possibly plasma globin or fibrinogen. The normal range for healthy young adults given by Wintrobe and Landsberg ('35) is 0 to 6.5 for men and 0 to 15 for women. Miller ('39) concluded that age had no effect upon sedimentation rates but Shapleigh, Mayes and Moore ('52) found that the rates of only 4% of the men and 8% of the women in their series of 100 subjects fell within the normal limits for the method they used. Forty per cent of the men and 60% of the women had sedimentation rates greater than 31 mm per hour. Olbrich ('48) also noted an increase with age, with mean of 12.1 mm per hour for males and 11.7 for females.

The mean for the 235 men over 50 years of age in this study was 12.6, for the 296 women 20.8. Twenty-four per cent of the women and less than 6% of the men had sedimentation rates of 31 mm per hour or more. Fifty-four per cent of the

men in our study and 36% of the women had sedimentation rates which fell within the normal range for young adults. The distribution of sedimentation rates for all the men and women examined is shown in figure 5. The rates noted for women are fairly evenly distributed among the 0–10, 21–30 and 31–50 values with a larger proportion in the 11–20 group.

TABLE 2

Sedimentation rate of blood of 531 men and women over 50 years of age

AGE GROUP IN YEARS, SEX	NO. OF SUBJECTS	SEDIMENTATION RATES				
		Mean	Range	Standard error	Median	"t" value of differences
		mm/hr.	*mm/hr.*		*mm/hr.*	
50–54						
Men	45	10	0.5–39	1.1	9	4.7
Women	49	19	3–46	1.5	17	
55–59						
Men	41	11	0–24	1.0	10	4.6
Women	55	18	1–39	1.2	17	
60–64						
Men	36	15	4–38	1.6	11	1.9 [1]
Women	57	19	2–39	1.3	16	
65–69						
Men	39	12	2–40	1.4	9	4.8
Women	57	22	1–47	1.6	22	
70–74						
Men	37	12	2–27	1.2	10	5.4
Women	40	24	4–49	1.9	23	
75–79						
Men	19	14	3–35	1.8	12	3.7
Women	25	24	7–41	2.0	24	
80 and over						
Men	18	20	4–59	3.3	19	1.3 [1]
Women	13	26	6–48	3.3	29	
Total						
Men	235	12.6	0–59	1.5	11	3.9
Women	296	20.8	1–48	1.5	18	

[1] Not significant at the 5% level.

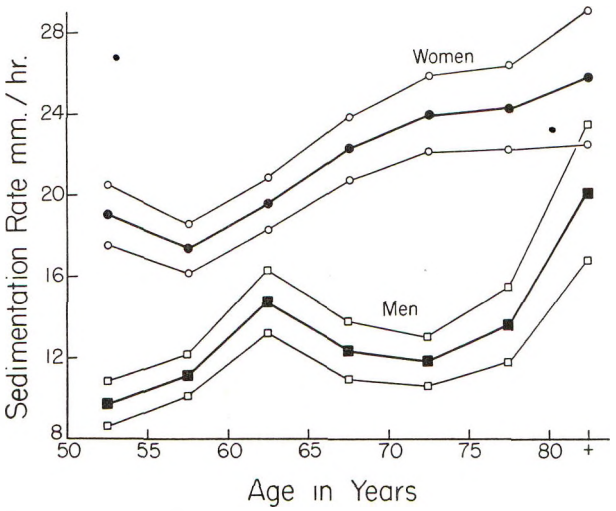

Fig. 4 Mean values and standard errors of blood sedimentation rates of men and women over 50 years of age.

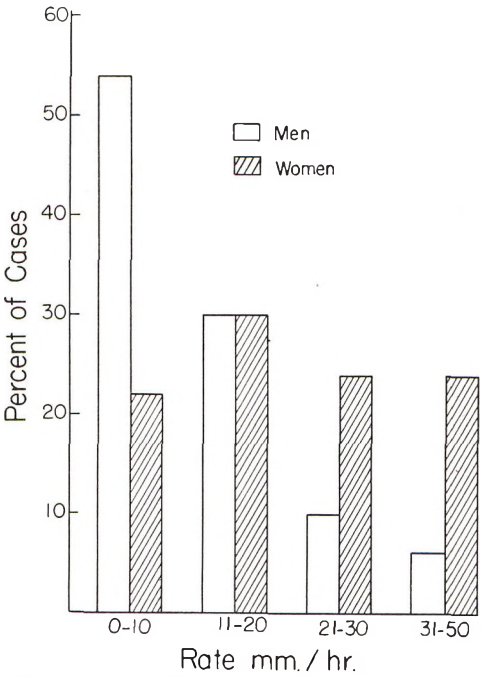

Fig. 5 Distribution of blood sedimentation rates in men and women over 50 years of age.

In the men the 0–10 rate was found in the large majority with regularly descending numbers in the higher rate groups.

Men in the county home. The hemoglobin, volume of packed cells and sedimentation rates of the blood of 44 men over 60 years of age living in the county home at Crystal Springs are set forth in table 3. Comparison with the corresponding values found in the blood of 149 men of similar ages living

TABLE 3

Hemoglobin, volume of packed cells and sedimentation rates of 44 men over 60 years of age living in the county home

BLOOD VALUE	AGE GROUP IN YEARS	NO. OF SUBJECTS	MEAN	RANGE	STANDARD ERROR
Hemoglobin	60–64	11	14.2	11.8–16.5	0.4
gm/100 ml	65–69	16	14.9	11.6–17.5	0.5
blood	70–74	8	14.1	11.6–16.2	0.7
	75 and over	9	14.2	11.8–16.8	0.8
Hematocrit,	60–64	11	43.6	35 –52	1.4
volume of	65–69	16	45.0	34 –53	1.5
packed cells,	70–74	8	42.3	34 –50	2.1
volume per cent	75 and over	9	42.3	35 –48	2.4
Sedimentation	60–64	11	8	2 –34	3.0
rate,	65–69	16	13	0.5–43	3.0
mm/hr.	70–74	8	22	4 –42	4.0
	75 and over	9	15	3 –47	11.0
Corpuscular	60–64	11	33		
hemoglobin	65–69	16	33		
concentration,	70–74	8	33		
per cent	75 and over	9	34		

in their own homes (tables 1 and 2) reveals that there were no significant differences in the means of hemoglobin concentration and sedimentation rates between the two groups except in the age group 70 to 74 years in which the men in the county home had definitely higher sedimentation rates. The volumes per cent of packed cells were consistently and probably significantly greater in the men living in their own homes than in those in the county home. This produced an

apparently greater corpuscular hemoglobin concentration in the latter group which may be due to smaller mean cell volumes. The ranges for hemoglobin and hematocrit tended to be narrower and the values more uniform in the men living in the county home than in those not institutionalized. If the values for these 44 men were included with those for the other 149 men of the same age groups, the mean hemoglobin would not be changed from 14.5 and the mean for volume of packed cells would be changed only from 47.1 to 46.4.

Relationship of dietary intake to hemoglobin concentration. It is generally thought that iron and amino acids are the most important nutrients for hemoglobin production. Direct evidence for this in the absence of marked hypochromic anemia and in any large sample of the population has not been available. The adequacy of the diet as to iron particularly has often been judged by the range of hemoglobin values found in any population group (Med. Res. Council, '45; Davidson et al., '35; Bethell et al., '34). The function of food protein in hematopoiesis is also well established experimentally but has not until recently been directly indicated in mass dietary and nutritional status studies. In particular, the relationship between iron and protein intakes of men and women and their prevailing hemoglobin levels is of considerable interest. Certain population groups, chiefly school children, college students, pregnant women and male industrial workers have recently been studied in 6 northeastern states with the resultant finding of significant correlations between hemoglobin and dietary protein and iron in nearly all groups (Babcock et al., '53).

The intakes of all the nutrients which were calculated in this survey were found to be uniformly greater for the men than for the women at all ages. The differences due to sex were more striking in certain cases than in others, for instance in calories, protein and iron, as shown in table 4. However the proportion of protein calories to total calories was almost exactly the same for all groups of men and women living at home, 14.0%. The iron intakes were expressed in milligrams

TABLE 4

Average daily intakes of calories, protein and iron of 533 men and women over 50 years of age

AGE GROUP IN YEARS, SEX	NO. OF SUBJECTS	CALORIES		PROTEIN			IRON		
		Mean	S.E.[1]	Mean	S.E.	% of calories	Mean	S.E.	per 1000 calories
				gm	*gm*		*mg*	*mg*	*mg*
50–54									
Men	40	2613	110	92	4	14.1	15	0.7	5.7
Women	45	1817	56	60	2	13.2	10	0.4	5.5
55–59									
Men	39	2624	95	95	4	14.5	16	0.6	6.1
Women	50	1742	59	62	3	14.2	11	0.5	6.2
60–64									
Men	34	2504	93	89	4	14.2	15	0.6	6.0
Women	55	1786	48	62	2	13.9	11	0.3	6.1
C.H. men[2]	11	1852	86	59	3	12.4	12	0.8	6.5
65–69									
Men	38	2370	87	80	3	13.5	14	0.6	5.9
Women	51	1815	49	64	2	14.1	11	0.4	6.0
C.H. men	16	1921	86	63	4	13.1	13	0.8	6.7
70–74									
Men	33	2138	58	76	3	14.2	13	0.5	6.0
Women	37	1685	58	65	3	15.5	11	0.5	6.5
C.H. men	8	1862	110	64	5	13.8	13	0.9	7.0
75–79									
Men	16	2160	127	77	5	14.2	15	1.0	6.9
Women	24	1514	72	53	3	14.0	9	0.6	5.9
C.H. men	5	1872	72	59	3	12.6	14	1.5	7.4
80 and over									
Men	15	2223	145	75	6	13.5	13	1.0	5.8
Women	12	1426	91	47	3	13.2	9	0.7	6.3
C.H. men	4	1865	138	63	6	13.5	11	0.8	6.0
Total									
Men	215	2376	102	83	4	14.0	15	0.7	6.0
Women	274	1683	62	59	3	14.0	10	0.5	6.1
C.H. men	44	1874	98	61	4	13.1	13	1.0	6.7

[1] Standard error.

[2] Men in county home.

per 1000 calories and were also similar, 6.0 for the men and 6.1 for the women (table 4). Protein and iron intakes by men and women were directly proportional to calories eaten which indicates similar composition of the foods.

The mean daily intakes per kilogram of body weight of protein and iron are shown in figures 6 and 7. It is evident

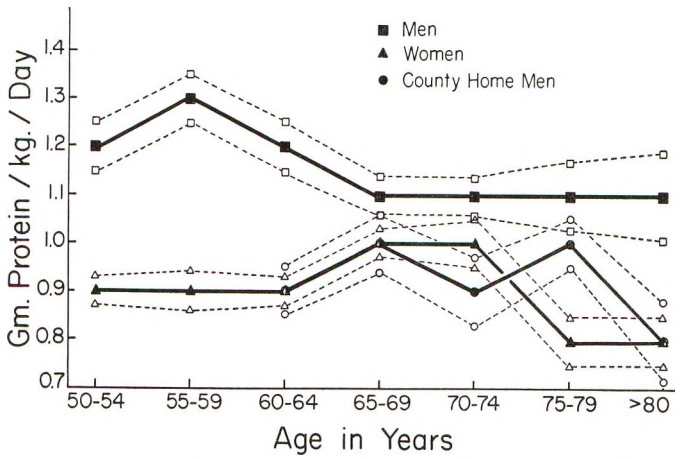

Fig. 6 Mean daily intakes and standard errors of protein per kilogram body weight as recorded by 235 men and 296 women over 50 years of age.

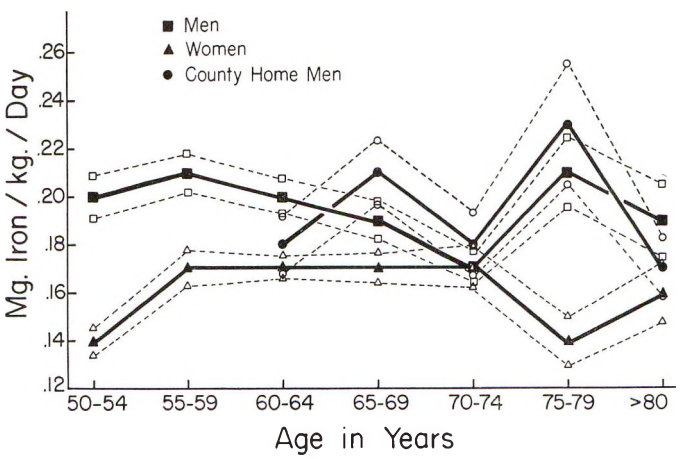

Fig. 7 Mean daily intakes and standard errors of iron per kilogram body weight as recorded by 235 men and 296 women over 50 years of age.

that for all age groups the intake of both nutrients was significantly lower for the women than for the men living in their own homes. As shown in table 4 the total calorie, protein, and iron intakes of the women were similarly less than that of the men. Their smaller average body size and probably lower muscular activity might seem to indicate lower requirements. When calorie, protein and iron intakes were expressed as values per kilogram body weight per day it was found that the intakes of the men living in their own homes were still significantly greater than those of the women. On this basis the calorie and protein intakes of the men in the county home were not significantly different from those of the women but their iron intakes were found to be equal to those of the men living at home and greater than those of the women (table 5).

The hemoglobin values of all cases were sorted as to protein intake and as shown in figure 8 the largest number of the lower hemoglobin levels was found in the group who had eaten the lowest amount of protein, 59 gm or less daily. Those in the medium intake group, 60 to 79 gm protein daily, had intermediate hemoglobin values and those who ate 80 gm a day or more had increasing proportions of high hemoglobin levels. The correlation statistic r was + 0.12 for the entire group, valid at the 1% level.

When a similar sorting of the iron intakes was done (fig. 9) a similar relationship emerged. The lowest iron intakes, 9.9 mg or less per day, represented a larger percentage of those with the lowest hemoglobin levels, the intermediate intakes, 10 to 13.9 mg per day, gave an intermediate range of hemoglobin figures, and the highest intakes, 14 mg per day or more, had the largest number of cases of high hemoglobin values. This distribution was the same in men and women. The correlation figure r was + 0.13 for the entire group, valid at the 1% level.

Although only one of the women in this survey had not completed the menopause, and in the youngest age group, 50 to 59 years, the average time elapsed since completion

TABLE 5

Intakes per kilogram of body weight per day of calories, protein and iron of 533 men and women over 50 years of age

AGE GROUP IN YEARS, SEX	NO. OF SUBJECTS	MEAN BODY WT.	CALORIES/KG/DAY		PROTEIN, KG/DAY		IRON, KG/DAY	
			Mean	S.E.[1]	Mean	S.E.	Mean	S.E.
		kg			*gm*		*mg*	
50–54								
Men	40	74.1	35	1.4	1.2	.05	.20	.009
Women	45	68.2	27	0.8	0.9	.03	.14	.006
55–59								
Men	39	74.5	35	1.3	1.3	.05	.21	.008
Women	50	65.9	26	0.9	0.9	.004	.17	.007
60–64								
Men	34	74.5	34	1.2	1.2	.05	.20	.008
Women	55	65.4	27	0.7	0.9	.03	.17	.005
C.H. men [2]	11	66.4	28	1.3	0.9	.005	.18	.012
65–69								
Men	38	70.9	33	1.2	1.1	.04	.19	.008
Women	51	65.0	28	0.7	1.0	.03	.17	.006
C.H. men	16	61.8	31	1.4	1.0	.06	.21	.013
70–74								
Men	33	70.9	30	0.8	1.1	.04	.17	.007
Women	37	65.6	26	0.9	1.1	.05	.17	.008
C.H. men	8	67.7	28	1.6	0.9	.07	.18	.013
75–79								
Men	16	70.5	31	1.8	1.1	.07	.21	.014
Women	24	63.2	24	1.1	0.8	.05	.14	.010
C.H. men	5	60.0	31	1.2	1.0	.05	.23	.025
80 and over								
Men	15	66.8	33	2.2	1.1	.09	.19	.015
Women	12	55.9	26	1.6	0.8	.05	.16	.012
C.H. men	4	64.5	29	2.1	0.8	.08	.17	.012
Total								
Men	215	72.4	33	1.4	1.1	.05	.20	.009
Women	274	65.0	26	0.9	0.9	.04	.16	.006
C.H. men	44	64.1	29	1.5	0.9	.06	.20	.014

[1] Standard error.

[2] Men in county home.

of the menopause was 8.2 years, the same lower hemoglobin values were found in this group and in the women of the older age groups as compared with men of the same age that have been recorded many times for young and middle-aged adult

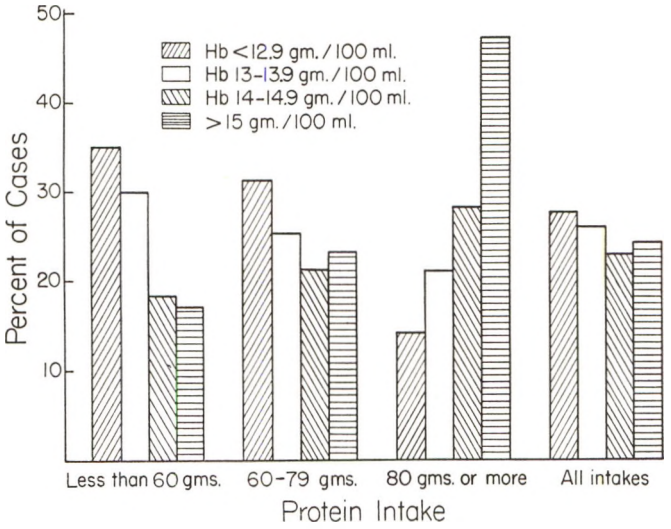

Fig. 8 Relationship of protein intakes to hemoglobin levels of men and women over 50 years of age.

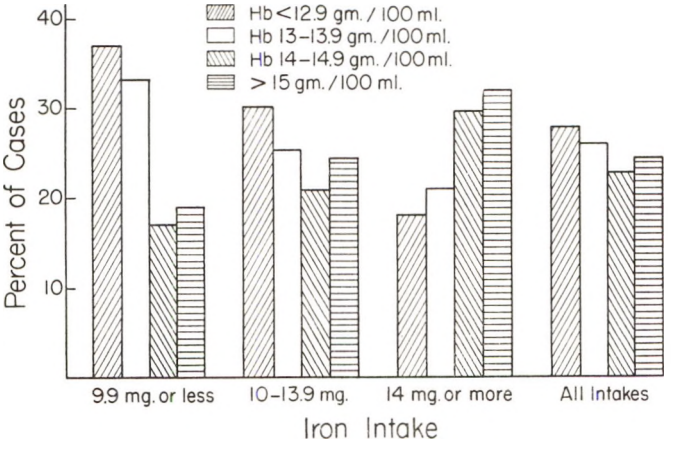

Fig. 9 Relationship of iron intakes to hemoglobin levels of men and women over 50 years of age.

men and women. The iron and protein intakes of these women in proportion to their total calorie intakes were fully equal to those of the men (table 4), but per kilogram of body weight they were slightly but not significantly less than those of the men (table 5). Yet both hemoglobin and hematocrit values remained significantly higher in the men until age 75. Beyond that age the differences tended to become insignificant. The possibility of a true sex difference in hematopoiesis should be considered. This has been suggested by work with hypophysectomized and castrated adult male rats (Crafts, '46). In the age groups beyond 75 years the male sex hormone production may have decreased enough to obliterate much of this difference. The difference in intake of both protein and iron per kilogram per day tend to become insignificant in the age groups between 65 and 74 years and to diverge significantly after 75 years (table 5). The hemoglobin levels also come close together in the age group 65 to 69 years but diverge at 70 to 74 years and approach again after 75 years. This might indicate other influences than those of intake, particularly in the oldest age groups.

To test further the hypothesis that a sex factor may influence hemoglobin production or destruction, or both, the intakes of protein and iron of all men and women with hemoglobin levels of 14 gm % or more were compared. As shown in table 6, at every level of intake the percentage of men supporting the 14 gm level was close to twice that of the women.

It may be noted in tables 4 and 5 and figures 6 and 7 that the 44 men in the county home had intakes of both protein and calories more like that of the women than the other men of like age. Their iron intake however was greater than that of the women and very close to that recorded by the other men. Likewise (table 3) they maintained hemoglobin levels fully equivalent to those of the men living in their own homes. This might point toward greater influence of dietary iron than of protein in maintaining hemoglobin production or to endocrine-influenced differences in the utilization of protein

by men and women. The quality of the protein eaten by the men in the county home may have been on the whole of somewhat lower biological value than that chosen by the majority of the other people studied.

Blood cells. There were 572 blood smear preparations which were examined for cytic morphology and color. Twenty-one per cent of the women and 14% of the men showed inequality in size of cells and of these about one-third malformed or

TABLE 6

Numbers of 254 men and 274 women having 14 gm hemoglobin per 100 ml blood or more with varying protein and iron intakes

DAILY INTAKES	TOTAL NUMBER		% HAVING 14 GM Hb % OR MORE	
	Men	Women	Men	Women
Protein, gm				
Less than 60	44	140	45	29
More than 60	210	134	73	25
More than 70	167	75	70	33
More than 80	118	38	71	42
More than 90	79	14	73	28
Iron, mg				
Less than 8.0	6	36	50	28
More than 8.0	249	238	69	23
More than 10.0	229	168	70	28
More than 12.0	187	87	70	29
More than 14.0	138	35	67	37
More than 16.0	82	15	68	27

oversized cells as well. So far as could be determined from a single observation there appeared to be two cases of leucopenia, two of polychromasia and 5 cases of increase in platelets among the men. Among the women 5% showed hypochromic normocytic and 2.5% macrocytic normochromic conditions. Among the men in their own homes there were two cases of hypochromic normocytic and two of microcytic normochromic anemia, 0.9% each. Thirty per cent of the 44 men in the county home had marked inequality in size of cells and most of these malformed or oversized cells as well. Ten

per cent showed microcytic normochromic and 10% microcytic hypochromic erythrocytes. One man had many macrocytes and one had definite leucopenia. The blood pictures of these men were significantly more abnormal than were those of the men living in their own homes.

SUMMARY

Physical examinations, medical and dietary histories, 7-day diet records and blood and urine analyses were carried out on 577 men and women over 50 years of age. More than 80% were in comfortable middle-class circumstances and all but 47 men were living in their own homes. These 47 men lived in the county home.

The hemoglobin levels of the men living in their own homes were higher than those of the women, 14.5 $\pm$ 0.3 gm per 100 ml of blood as compared with 13.4 $\pm$ 0.3. In the groups 75 years of age and older however, the values declined in both men and women and the difference became insignificant. The mean values were 1 to 2 gm % lower than those usually reported for young adults but the discrepancy was greater in men than in women.

The volume of packed cells was likewise larger in men than in women, 47.1 $\pm$ 0.7% in men living in their own homes and 44.6 $\pm$ 0.5 in women. These values are equal to those usually quoted as normal for young adults. The mean corpuscular hemoglobin concentration for these men and women was 31 and 30%, somewhat lower than in young adults, and due to the apparently increased cell size in the older subjects.

The sedimentation rates were higher in women than in men and tended to increase with advancing age. Eighty-four per cent of the men and 52% of the women had rates 0 to 20 mm per hour, and 16% of the men and 48% of the women 21 to 50.

The diet records indicated that the men living in their own homes ate from 15 to 34%, mean 27, more calories and protein and 0 to 50%, mean 26, more iron per kilogram body weight than the women in the same age groups. The protein calories were 14% of the total in both sexes and the iron intake 6.0

and 6.1 mg per 1000 calories. The qualitative nature of the diets was apparently the same. A trend toward decrease with age in intake of these nutrients was evident.

The majority of the higher intakes of both protein and iron were found in the groups with higher levels of hemoglobin, the intermediate intakes in those with intermediate hemoglobin levels and the lowest intakes in those with the lowest hemoglobin values. The positive correlation between dietary protein and hemoglobin was + 0.12 and between iron and hemoglobin + 0.13, both significant at the 1% level.

The men in the county home exhibited hemoglobin concentrations similar to those of the men living in their own homes but the volume of packed cells was somewhat lower. The protein intake of these men was of the same order as that of women but their iron intake was equal to that of the other men. This may point to greater importance of dietary iron than protein in regard to hemoglobin production or to a sex difference in this function. Although these women had comparable intakes of protein and iron in proportion to total calories and were in all but one case past the menopause, they exhibited low hemoglobin levels in comparison with men of the same ages and general circumstances. At every level of protein and iron intake the percentage of men supporting a circulating level of 14 gm % or more of hemoglobin was about twice that of the women. There is a possibility that the male sex hormone may influence blood production significantly at least up to 75 years of age.

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NUTRITIONAL STATUS OF THE AGING [1]

II. BLOOD GLUCOSE LEVELS

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FOUR FIGURES

(Received for publication May 10, 1954)

In a study of the nutritional status of 577 supposedly healthy men and women over 50 years of age in San Mateo County, California, 7-day diet records, diet histories, physical examinations and blood and urine analyses were obtained. Forty-five of the men were living in the county home, all others in their own homes. The main purpose was to discover any relationships that might exist between health status and nutriture and nutrient intake. Blood glucose levels were determined since some doubt exists as to the normal range of this constituent in elderly persons. This problem has been reviewed recently by Smith ('48) and by Shock ('52).

METHOD

The procedure was chosen so as to provide a maximum margin of safety against exaggeration of the blood glucose concentrations. Venous blood was obtained because its sugar level is known to be lower than that of capillary or arterial

[1] This was part of the Western Regional Cooperation Project, W-4, on nutritional status of population groups. It was supported in part by funds appropriated under the Research and Marketing Act of 1946. Effective cooperation was given by 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.

blood and its return to normal or lower than normal post absorptive levels after ingestion of carbohydrate faster than in the latter (Foster, '23). The analytical method used reduced to a minimum the measurement of reducing substances other than glucose. This was a modification of the method of Miller and Van Slyke ('36) combined with the ceric sulfate titration method of Giragossintz, Davidson and Kirk ('36). The blood was deproteinized with copper sulfate and sodium tungstate (Somogyi, '30), reduced by ferricyanide and reoxidized by titration with the standardized ceric sulfate solution. Two milliliters of blood from each subject provided for duplicate or triplicate determinations. Unless the analysis was performed within a few minutes after the sample was drawn the blood was kept frozen for analysis the next day.

The plan involved taking of blood samples two hours or more after the last meal. The exact period since the last meal was recorded for each subject at the time the sample was taken. The subjects were given full directions as to the composition of the meal, which was to consist of carbohydrate food chiefly, with little fat or fruits and vegetables. The venous blood glucose in young adults usually has returned to the postabsorptive level or lower in this period after a meal.

The urine was tested for reducing sugar by adding 8 drops to 5 ml of Benedict's qualitative copper reagent which was left in the boiling water bath for 5 minutes and the findings reported as a trace, that is cloudiness after cooling, and 1 +, 2 +, 3 +, 4 + for increasing amounts of precipitation.

RESULTS

In table 1 the results are recorded by age and sex for the men and women living in their own homes, with segregation of all values above 130 mg per 100 ml of blood. It is obvious that a considerable range was found with no clear differences due to age or sex although there is some tendency toward an increase in the levels of the women, after 75 years of age, but not of the men. The distribution is shown in figure 1.

Sixty-five per cent of the women and 59% of the men had glucose levels between 90 and 109 mg per 100 ml of blood. The values show a normal distribution between the extremes of 55 and more than 130.

TABLE 1

Blood glucose of men and women over 50 years of age, two hours or more after the last meal

AGE	NO. OF SUBJECTS	MEAN (all under 130 mg)	RANGE	STANDARD ERROR	NO. ABOVE 130 MG/100 ML	GLYCOSURIA AT ALL BLOOD LEVELS
years		*mg/100 ml*	*mg/100 ml*			*no. of cases*
50–54						
Men	25	106	67–130	2.9	3	5 (1+,1++) 3 tr.
Women	34	97	76–129	1.4	0	1 tr.
55–59						
Men	23	104	77–128	6.2	1	4 (1++++) 3 tr.
Women	43	96	60–124	3.2	3	3 tr.
60–64						
Men	26	93	55–121	2.4	1	3 (1+) 2 tr.
Women	39	97	62–128	1.9	0	1 (1++++)
65–69						
Men	24	107	59–130	5.1	2	3 (1++) 2 tr.
Women	48	102	74–127	2.1	3	0
70–74						
Men	22	107	57–130	8.4	2	3 (1++++) 2 tr.
Women	28	102	78–122	2.6	2	0
75+						
Men	27	103	63–124	3.5	3	1 (1+)
Women	27	116	90–128	14.8	2	3 (1+,1+++,1++++)
Totals						
Men	147	103.1	55–130	4.6	12	19
Women	219	100.9	60–129	3.8	10	8

Blood glucose levels were determined in 45 men living in the county home, and values for 42 of these are presented in table 2. Three determinations were omitted because the men had eaten food less than two hours before the sample was taken. It is obvious that the blood sugar levels, except for the

men under 65, are significantly lower in this group than in others of the same age groups living in their own homes (table 1). Three of the 6 men who had high blood sugar levels had glycosuria and 4 others with normal or low blood sugar also had glycosuria. Two years after the examination 5 of these men who had had hyperglycemia or glycosuria or both were dead and after three years a 6th man had died. The cause of death given in the three cases in which a report was available was arteriosclerosis in some form.

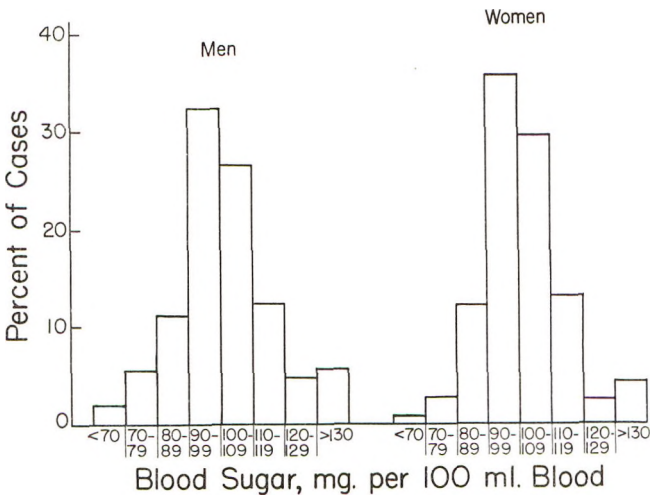

Fig. 1 Distribution of blood glucose levels in men and women over 50 years of age, two hours after the last meal.

Effect of time interval

In all there were 92 men and 74 women who had eaten the last meal less than two hours before the blood sample was drawn. The blood levels of these 167 people are not included in table 1 but are presented in figure 2 along with the others in an attempt to discern the relationship of this time interval to the blood glucose concentration. It is obvious that values under 70 mg are few in number, none being found in samples taken less than two hours after a meal and only two or 3% of the total in those taken two hours after a meal.

Values between 70 and 99 mg% increase steadily from 27% of total cases after one to one and one-half hours to 58% after three hours. A steady proportion of 42 to 49% of all the cases were in the range 100 to 130 mg% when the last meal was taken one to three hours before the sampling. There were

TABLE 2

Blood glucose of men in the county home two hours or more after the last meal

AGE	NO. OF MEN	MEAN (of those 130 mg or less)	RANGE	STANDARD ERROR	NO. ABOVE 130 MG	GLYCOSURIA AT ALL BLOOD LEVELS
years		*mg/100 ml*	*mg/100 ml*			*no. of cases*
50–54	1	116				1 tr.
55–59	1	96				
60–64	11	98	66–121	6.5		
65–69	11	84	59– 93	4.4	5	2 (1+) 1 tr. 1 (1++++)
70+	12	86	57–118	5.7	1	2 tr.
Total	36	90	57–121	5.2	6	7

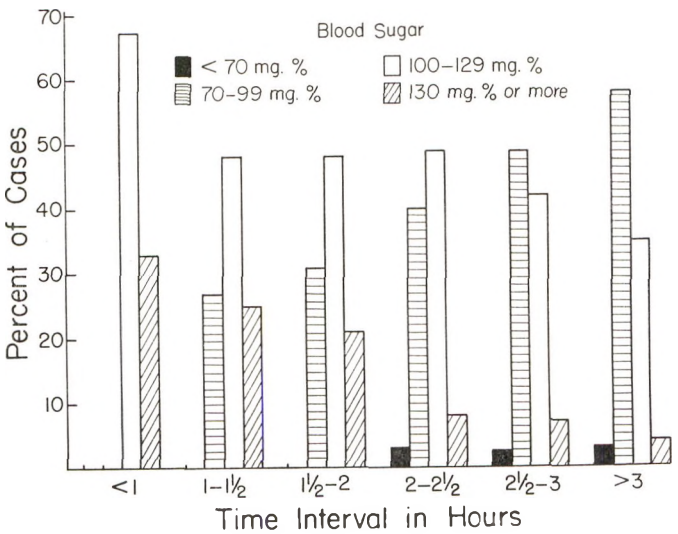

Fig. 2 Effect of interval since last meal on distribution of blood sugar levels.

values over 130 mg% at all time intervals but the percentage of cases fell from 33 at one hour or less to 4 after three hours.

Hyperglycemia and glycosuria

The distribution of blood sugar levels of 73 of the subjects is illustrated in figure 3. The blood sugar values are shown for all subjects who had a glucose level of 130 mg or over who had shown any evidence of glycosuria. The blood sugar

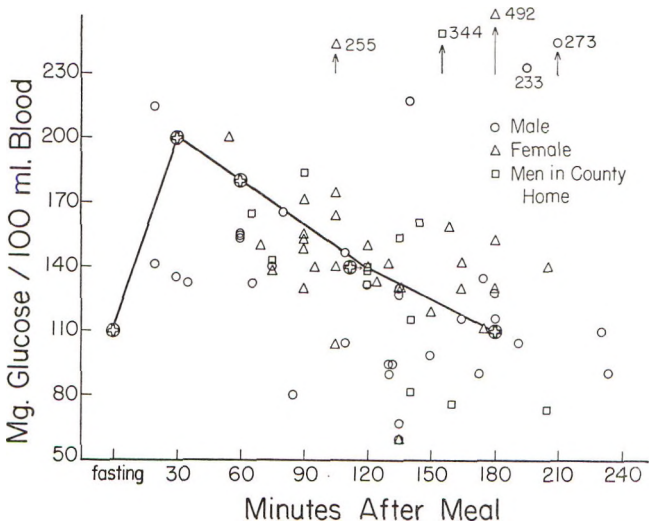

Fig. 3 Blood glucose levels of 73 men and women with signs of hyperglycemia or glycosuria or both or irregular time intervals since the last meal.

levels of the men in the county home whose records fell in these categories are also included in figure 3. These 73 cases were chosen for illustration because they represent unusual or abnormal sugar tolerance values, or because of the variety of time intervals since the last meal was eaten. All suspected diabetics are included in this group.

Various postprandial blood sugar levels have been suggested as hyperglycemic. The time-blood sugar line curve used by McCullagh and Zwickel ('53) as indicative of hyperglycemia is shown in figure 3. Twenty-nine of the 73 cases

depicted in figure 3 fall on or above this line. Eleven are above 170 mg per 100 ml (Folin-Wu method) suggested by Wilkerson and Krall ('53) as hyperglycemic for venous samples and 10 are above 150 after two or more hours, a criterion of diabetes suggested by Moyer and Womack ('48). On the basis of these various blood sugar criteria the incidence of hyperglycemia in this population sample was 2.1 to 5.6% for men and 1.3 to 4.4% for women. Of these 10 to 29 persons suspected of hyperglycemia by various standards only 5 had a history of diabetes known before the examination. Seven others who reported a diagnosis of diabetes in the past had neither hyperglycemia nor glycosuria. Only one of the 8 persons who had a family history of diabetes was diabetic.

There were 38 persons with blood sugar levels over 130 mg who showed no glycosuria. Of these, 16 had had no food for more than two hours before the sample was drawn. Of the 22 found to have a "trace" of sugar in the urine, 15 had blood sugar levels under 130 mg and 7 were over that level. Glycosuria designated as of the order 1 + to 4 + was found in 10 people with blood sugar over, and 4 under 130 mg per 100 ml of blood. If the criteria of 1 + to 4 + glycosuria and blood sugar above 130 mg two hours after a meal be used, there were 8 men and two women in this group who were probably diabetic. On this basis the rate is 3.0% for men and 0.7% for women (table 3).

Because of the probable variation of renal reabsorption efficiency in elderly people the expected correlation between blood sugar levels and glycosuria is less reliable than has been found in younger people. Sustained high blood sugar levels may occur without glycosuria and measurable glycosuria when a normal blood sugar level is present.

The mortality rate of the whole group examined was recorded 5 years after the study. The highest incidence of hyperglycemia was found among the men in the county home and also the highest mortality rate, 31%. The mortality rate for all of the men under 70 years of age including those in the county home was 11.6%, and of those over 70 years, 25.3.

TABLE 3

Incidence of hyperglycemia and glycosuria in 575 men and women over 50 years of age

AGE GROUP	MORE THAN 2 HRS. POSTPRANDIAL		LESS THAN 2 HRS. POSTPRANDIAL SHOWING GLYCOSURIA
	Blood sugar	Glycosuria	Blood sugar
years	*mg/100 ml*		*mg/100 ml*
50–59			
Men	142	none	
	67,116,128,130	trace	140,154,214,80
		1+	147
	135	2+	
	232	4+	
Women	142,153	none	
	60,111	trace	104,142
60–69			
Men	160,140,132	none	
	99,132,127	trace	191,110
	76,154,116	1+	
	344,217	4+	
Women	132,133,150	none	
		4+	255
70–79			
Men	132,159,143,139	none	
	110,116,81,73	trace	
	273	4+	
Women	140,140	none	
	128	1+	
	118	3+	
	492	4+	
80 +			
Men	141	none	
		1+	233
Women		trace	163
Total	16	none	
	13	trace	9
	11	1+ to 4+	3

The mortality rate for women under 70 years was 3.5% and over that age, 11.4. Persons with blood sugar level under 100 mg% showed a mortality rate of 7.1, with levels of 100 to 129, 7.6, and in those with levels over 130 mg% the rate was 16.6.

The incidence of true diabetes in the elderly population examined during this study cannot be established since in most cases no effective follow-up was conducted to confirm the diagnosis. However, a few suggestions emerge. In an elderly group particularly, blood sugar and urine sugar tests do not always agree. In this group 28 individuals had blood sugar above 130 mg two hours or more postprandial, and 10 of these also had a moderate degree of glycosuria. But 16 of these subjects with equally high blood sugar had no sugar in the urine. Of 36 persons with signs of glycosuria including 12 persons who had eaten a meal less than two hours before the examination, 18 had normal or low blood sugar (table 3). The safest generalization that can be drawn from this study is that in people over 50 years of age, hyperglycemia may occur in about 5%, glycosuria of some degree in about 6% and both of these signs in nearly 2%.

Relationship of blood glucose to dietary intake

Since it has been shown (McCullagh and Johnston, '38; Livingston and Bridge, '42) that the habitual intake of carbohydrate and fat may affect the slope of the glucose tolerance curve, an effort was made to determine whether any correlation existed between the 7-day dietary records of the participants in this study and their blood sugar levels. These dietary records were taken and calculated for nutrient contents as previously described (Gillum and Morgan, '55). The mean calories, protein, carbohydrate and fat in the food eaten by all the subjects are shown in table 4. As was true of nearly all of the nutrients the men at every age ate more total food, more protein, fat and carbohydrate than the women. The men in the county home had fat and protein in-

takes similar to those of the women but carbohydrate intakes more nearly approaching those of the other men. After 70 years of age the carbohydrate intake of the men in the county

TABLE 4

Protein, fat and carbohydrate intakes of men and women over 50 years of age

AGE GROUP	NO. OF SUB-JECTS	TOTAL CALORIES PER DAY		PROTEIN GM/DAY		FAT GM/DAY		CARBOHYDRATE GM/DAY	
years		*Mean*	*Standard error*	*Mean*	*Standard error*	*Mean*	*Standard error*	*Mean*	*Standard error*
Men									
50–54	40	2613	110	92	4	109	5	285	15
55–59	39	2624	95	95	4	113	5	285	13
60–64	34	2504	93	89	4	101	5	282	9
65–69	38	2370	87	80	3	97	5	277	11
70–74	33	2138	58	76	3	88	3	256	9
75–79	16	2160	127	77	5	85	6	254	16
80 +	15	2223	145	75	6	93	7	261	20
Women									
50–54	45	1817	56	60	2	76	3	210	8
55–59	50	1742	59	62	3	71	3	209	7
60–64	55	1786	48	62	2	75	3	211	6
65–69	51	1815	49	64	2	76	3	216	6
70–74	37	1685	58	65	3	71	3	196	8
75–79	24	1514	72	53	3	60	3	189	11
80 +	12	1426	91	47	3	55	4	183	14
Men in County Home									
60–64	11	1852	86	59	3	67	4	254	14
65–69	16	1921	86	63	4	74	4	251	14
70–74	8	1862	110	64	5	71	4	242	16
75 +	9	1868	137	61	5	67	7	255	21

home was not significantly different from that of men living in their own homes.

The subjects of all ages, separated as to sex, were divided into three groups according to their blood sugar levels; (a) those having less than 100 mg per 100 ml blood, (b) those having 100 to 129, and (c) those having more than 129. Each of these three groups was then sorted as to fat intake into

three subgroups; (a) those ingesting less than 60 gm of fat per day, (b) those ingesting 60 to 79, and (c) those ingesting more than 80. These three groups in the case of the women were almost equal in number. The resulting percentages found in the records of the women are shown in figure 4. There was an indication of a decrease in the percentage of women with the lowest blood sugar levels as the fat intakes increased and a small increase in the percentage of high blood sugar

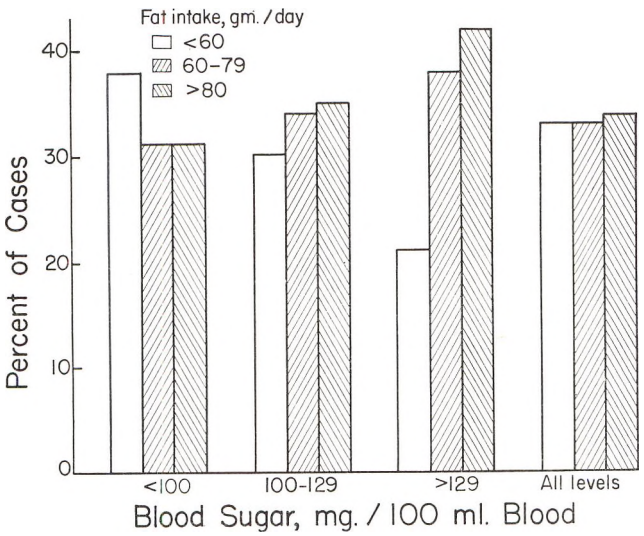

Fig. 4 Distribution of blood glucose levels of women in relation to average daily intake of fat.

levels with increased fat intakes. A corresponding relationship did not appear when the records of the men were compared. The fat intakes of the men fell into almost equal groupings, at less than 80, 80 to 109 and more than 110 gm per day. There were no comparable relationships seen when a similar sorting was made as to carbohydrate and protein intakes.

DISCUSSION

The blood glucose figures may be compared with the post-absorptive blood sugar levels observed by Punschel ('23) in

15 subjects 58 to 70 years old, 106 mg per 100 ml, and in 15 cases over 70, 110. The height of hyperglycemia after ingestion of 20 gm of glucose in these two groups was reached in 90 to 109 minutes respectively. However, Punschel used Bang's method which records other reducing substances besides glucose. Lozner et al. ('41) found no rise in blood sugar with age in their series of 60 presumably normal persons. Only 14 of these subjects however were over 50 years of age. In neither of these studies were the numbers and sex and age distribution of the subjects sufficiently broad to represent the ranges to be expected in a free-living population.

As shown in figure 1, 65% of the women and 59% of the men in our study had blood sugar levels in the range 90 to 109, and the values for 90% of the women and 82% of the men fell in the range 80 to 119. In the Lozner et al. ('41) study only 15% of the subjects had more than 100 mg% of blood sugar whereas in our study more than 49% were in this range. In the study by Hale-White and Payne ('26) of 12 healthy men 60 to 75 years old, it was found that in two hours after ingestion of 50 gm of glucose the blood sugar value had not returned to the fasting level but varied between 120 and 200 mg%. In 11 men under 30 years of age all had blood sugar values equal to or less than the fasting levels under similar circumstances.

The blood glucose range usually given in the older textbooks is 80 to 120 mg per 100 ml of blood (by the Folin-Wu method). However, a compilation based on the more accurate modern methods lists 76 to 96%, mean 86, for postabsorptive values of venous blood in man (Albritton, '52). This is based chiefly on the work of Somoygi ('48) and represents the 95% range of such values. Similar examination of our results (fig. 1) indicates that the 95% range for men and women over 50 years of age is 80 to 129, mean 101. Since these were determinations on postprandial rather than fasting blood samples, the higher levels may represent chiefly a slow return of the sugar to the fasting levels. The low blood sugar levels seen in 23 of the men over 65 years old in the county home

are puzzling, particularly in view of the glycosuria present in three of them. Six others of these men were hyperglycemic and three of them were glycosuric as well.

SUMMARY

Of 577 presumably normal men and women over 50 years of age examined as to dietary intake, physical condition and blood and urine composition, postprandial blood sugar levels of 430 are reported. All records used in computation of means were of persons who had eaten a carbohydrate meal two hours or more previous to withdrawal of the venous blood sample. Forty-five men were living in the county home and the rest of the subjects in their own homes.

The mean venous blood sugar levels, excluding all over 130 mg per 100 ml, determined by a method which recorded the true reducing sugar content, calculated for 5-year age intervals and separated as to sex showed little variation with age or sex, although there was a slight indication of an increase with age in the women. The overall mean was 101 mg per 100 ml of blood. This is significantly higher than the means usually quoted for young adults.

Hyperglycemia judged by various criteria was found in 2.1 to 5.6% of men and 1.3 to 4.4% of the women.

Glycosuria of varying degrees was noted in 36 cases, 24 of them two hours or more after the last meal. In 18 of these cases the blood sugar level was over 130 mg per 100 ml of blood and in 18 it was under that amount. Moderate to severe glycosuria and hyperglycemia occurred together in 8 men and two women. Four women and 14 men had equally severe glycosuria but normal or low blood sugar levels. Only 5 of these persons were known previously to be diabetic. Variable renal reabsorption of glucose may account for the discrepancies in blood and urine findings.

The effect on distribution of the blood sugar levels of food intake at one-half hour intervals previous to the withdrawal of the blood sample was noted in a group of 167 persons.

The men in the county home over 65 years of age had significantly lower blood sugar levels, mean 85 mg per 100 ml of blood, than men of the same age living in their own homes. There were 6 hyperglycemic and three diabetic cases also in the county home group. This group presented an unusual incidence of diabetes and its presence in the sample may account for the apparent higher incidence of these signs in men than in women.

A moderate positive correlation between fat intake and blood sugar level was found in the women but not in the men. No correlation of blood sugar with carbohydrate or protein intake was found in either men or women.

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THE VANDERBILT COOPERATIVE STUDY OF MATERNAL AND INFANT NUTRITION

VII. TOCOPHEROL IN RELATION TO PREGNANCY [1]

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ONE FIGURE

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The role of tocopherol in human nutrition is undefined, and studies of the influence of this vitamin on human pregnancy have been indecisive. Few estimates exist of the human dietary intake of vitamin E. Several reports of the levels of total tocopherols in the blood during pregnancy agree that a rise occurs as gestation progresses. For example, Straumfjord and Quaife ('46) noted an increase in the mean serum level from 1.17 mg/100 ml during the first 24 weeks of gestation to 1.62 mg/100 ml in the 25th to 36th week. Similar changes have been reported by Darby, Cannon and Kaser ('48), Scrimshaw, Greer and Goodland ('49), Varangot ('42), and Rauramo ('47). No attempts to correlate blood levels and dietary intake of tocopherol during pregnancy have come to our attention.

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The present communication reports the results of 1,575 determinations of plasma tocopherols on 1,572 [2] pregnant women and estimates of dietary tocopherol intake for 197 of these women. A separate group of 39 women, not otherwise participating in the study, had serial determinations of plasma tocopherol during pregnancy and for 35 of these subjects estimates were made at the 6-week postpartum examination. The relation of plasma tocopherol levels to the clinical course of pregnancy is explored.

METHODS

The Vanderbilt Cooperative Study of Maternal and Infant Nutrition has been described (Darby et al., '53a, b; McGanity et al., '54a, b). Estimations of total plasma tocopherols were made by the method of Quaife and Harris ('44) as modified by Quaife and Biehler ('45), on 1,575 of the 2,129 entrants in the study. Failure to obtain determinations on all patients was due to several factors, none of which is believed to have produced bias. These factors are as follows: The study of tocopherol was initiated after the other portions of the investigation were under way; the blood samples for this determination were obtained on the third clinic visit, and a few patients did not make as many as three visits; and during a period when radioactive iron metabolism studies (Hahn et al., '51) were being made, a single sample of blood served for both the tocopherol and radioactivity determinations, priority being given to the latter when the quantity was limited.

Thirty-five pregnant white women consecutively entering the Vanderbilt Hospital Outpatient Clinic constituted a minor study group. This group had repeated measurements of tocopherol content of plasma at intervals of 4 to 6 weeks from the time of entry into the study through the 6-week post-

[2] Henceforth the three determinations on duplicate subjects in the major series will be treated as though they are independent and we shall define the total number of determinations (1,575) as the number of "individuals" in the group.

partum examination. This series is not a portion of the major series of 1,575 patients.

Calculations of tocopherol content of the diets were based upon the 7-day dietary records obtained in the study. These calculations were made on a sample of 197 dietaries collected from May, 1948, from subjects upon whom plasma tocopherol values were available. The values for the vitamin E content of foods reported by Harris, Quaife and Swanson ('50) formed the basis for the estimation of intakes. The foods were grouped as usual (Steinkamp, Robinson and Kaser, '45), except that margarine, mayonnaise, other salad dressings and sweet potatoes were listed individually because of their high tocopherol content. It was assumed that cookies and cakes were made with vegetable shortening.

FINDINGS

Dietary intake. The calculated daily intakes of total tocopherol ranged from 2.9 to 33.3 mg. Only total tocopherol was calculated because of the less complete information concerning the distribution in foods of α-tocopherol. It may be estimated that α-tocopherol constituted some 50 to 60% of this total.

These estimates of intake approximate the averages calculated by others for various diets. Thus, Harris, Quaife and Swanson ('50) calculated from average per capita food consumption data a total tocopherol intake of 24 mg/day, 59% of which was α-tocopherol. A diet of 1,560 calories which followed the pattern of the Recommended Dietary Allowances provided 7.59 mg of total tocopherol, 75% of which was α-tocopherol (Quaife et al., '49). These same investigators (loc. cit.) calculated a range of α-tocopherol intake of from 5.7 to 12.8 mg/day for a small group of pregnant women in Rochester, New York. Accordingly, it would appear that our subjects did not differ greatly from a widespread pattern of intake of vitamin E within the United States.

Tabulations by season are shown in table 1; no striking variation is apparent. The slightly higher levels of intake

during winter were related to a high consumption of cookies and cakes by a few subjects.

Plasma tocopherol. The average tocopherol contents of plasma increased from 0.89 ± 0.02 mg/100 ml for measurements during the first trimester to 1.40 ± 0.05 mg/100 ml at 39 or later weeks of gestation (table 2). Variabilities increased along with the mean values and in such proportion that the coefficient of variation remained fairly constant. Furthermore, an increased concentration of tocopherol was observed

TABLE 1

Distribution of total tocopherol intakes (mg/day) by season for 197 pregnant women

TOCOPHEROL INTAKE	WINTER	SPRING	SUMMER	FALL	TOTAL
mg/day					
< 4	0	0	1	1	2
4–7	12	4	28	14	58
8–11	21	5	25	22	73
12–15	12	0	17	10	39
16–19	3	0	6	5	14
≥ 20	8	1	1	1	11
Total	56	10	78	53	197
VALUES OF					
1st quartile	8.2	6.8	7.3	7.8	7.5
Median	10.7	8.9	9.8	9.9	10.0
3rd quartile	14.6	10.8	12.4	13.1	13.2

with age (table 3 and fig. 1). This age effect is consistent with findings of a previous study of cardiac patients in this laboratory (Lemley et al., '49) and of Chieffi and Kirk ('51) for men. No difference associated with parity could be detected below para 5 (table 3). The group of para 5 or over had, after allowance for their age distribution, slightly lower average values. This small difference may be related to intake level or other factors associated with economic status (Harris et al., '46) and not to parity *per se.*

Relation of plasma tocopherol level to tocopherol intake. Plasma levels of tocopherols for women calculated to have

lower intakes were slightly below those of women with higher intakes (table 4), but the difference was small in comparison to individual variability in plasma values at any one intake level. In the second trimester, the group with intakes below 8 mg/day had significantly lower plasma tocopherol levels

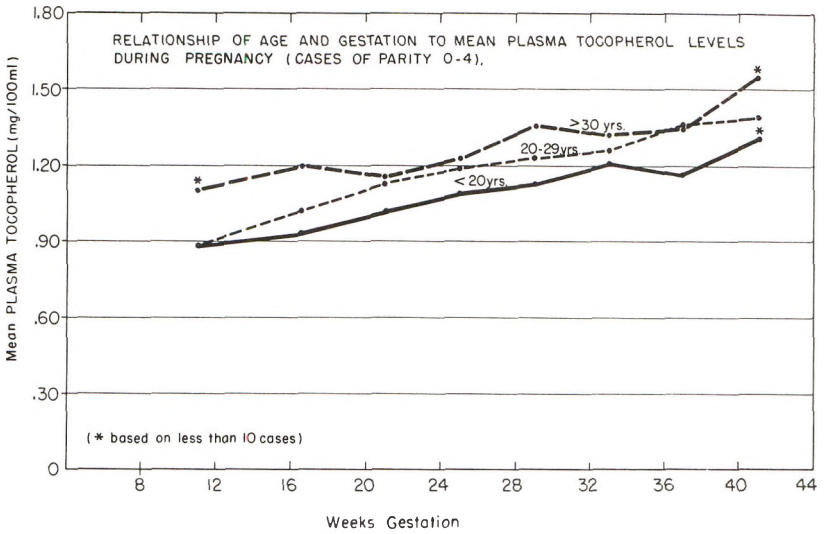

Figure 1

TABLE 2

Plasma tocopherol levels (mg/100 ml) by weeks of gestation for 1575 pregnant women

WEEKS GESTATION		NO. OF CASES	PLASMA TOCOPHEROL (MG/100 ML)		
Range	Median		Mean ± S.E.[1]	S.D.[2]	C.V.[3]
					%
8–13	11.5	74	0.89 ± .024	.20	23
14–18	16.5	240	1.04 ± .015	.23	22
19–22	21.0	258	1.09 ± .014	.23	21
23–26	25.0	273	1.16 ± .015	.24	21
27–30	29.0	273	1.21 ± .016	.26	22
31–34	33.0	276	1.26 ± .015	.25	20
35–38	37.0	149	1.32 ± .024	.29	22
39–42	41.0	32	1.40 ± .050	.28	20

[1] S.E. — standard error of the mean.

[2] S.D. — standard deviation.

[3] C.V. — coefficient of variation (S.D./mean).

than did the group with intakes of 12 mg/day or over or than did the combined group with intakes of 8 mg/day or over; non-significant differences in the same direction occurred in the data for first and third trimesters.

Relation of plasma tocopherol to other laboratory values. Plasma tocopherols and other laboratory determinations were not usually done on simultaneous samples, and so study of interrelations is limited. Only for serum carotene could there be found a relationship to plasma tocopherol levels; no relation was noted to serum content of vitamin A, ascorbic acid, or any other nutrient (Darby et al., '53b).

TABLE 3

Plasma tocopherol levels in each trimester of pregnancy, by age and parity

PARITY	AGE	PLASMA TOCOPHEROL (MG/100 ML)					
		First trimester		Second trimester		Third trimester	
		Number	Mean ± S.E.	Number	Mean ± S.E.	Number	Mean ± S.E.
	years						
0–4	14–19	12	0.88 ± .06	205	1.02 ± .01	164	1.17 ± .02
0–4	20–24	28	0.89 ± .03	254	1.10 ± .01	250	1.26 ± .02
0–4	25–29	21	0.85 ± .04	134	1.16 ± .02	103	1.31 ± .03
0–4	≥ 30	6	1.10 ± .07	77	1.18 ± .03	80	1.35 ± .03
5 +	< 30	2	0.70 ± .20	19	1.17 ± .07	28	1.23 ± .05
5 +	≥ 30	2	0.70 ± .20	73	1.14 ± .04	88	1.31 ± .03

The association of blood concentrations of tocopherol and carotenes, albeit weak, was evident in all comparisons made at successive stages of gestation, whether the carotene determinations preceded the tocopherol determinations (table 5) or followed later in pregnancy or postpartum (table 5). It is not meant to imply that there existed a direct causal action of one or the other; more likely, this similar behavior is evidence of metabolic factors which tend to influence both carotene and tocopherol levels in the serum (Darby, Cannon and Kaser, '48).

Successive serum carotene measurements on individuals at progressive stages of pregnancy and postpartum had coefficients of correlation of the order of 0.5; and a limited

group of tocopherol levels during the last month of pregnancy compared with postpartum levels showed a correlation coefficient of 0.6. These values indicate a considerable individual consistency of level, and suggest that the relationship of the two occurs outside of pregnancy. The strength of relationship noted in specific groups under study will be

TABLE 4

Relation of plasma tocopherol level to total tocopherol intake by trimester of pregnancy (mean ± S.E. and standard deviation of plasma tocopherol values for three intake levels), 197 cases

TOCOPHEROL INTAKE	PLASMA TOCOPHEROL (MG/100 ML)		
	No. of cases	Mean ± S.E.[1]	S.D.[2]
mg/day			
First trimester:			
Under 8.0	9	0.97 ± .068	.20
8.0–11.9	13	1.06 ± .083	.30
12.0 and over	10	1.08 ± .088	.28
Second trimester:			
Under 8.0	25	1.02 ± .035	.18
8.0–11.9	28	1.10 ± .038	.20
12.0 and over	33	1.19 ± .042	.24
Third trimester:			
Under 8.0	26	1.18 ± .057	.29
8.0–11.9	32	1.28 ± .053	.30
12.0 and over	21	1.28 ± .055	.25

[1] S.E. = standard error of the mean.
[2] S.D. = standard deviation.

influenced by: the variability of true levels of the two nutrients, errors of method in determining levels, possible genetic factors which control normal values of various nutritional factors (Sutton and Vandenberg, '53), and whatever factors (intake, blood volume changes, etc.) are responsible for changes in concentrations from time to time. Numerous studies in experimental animals have established that the level of tocopherol intake can influence efficiency of carotene utilization as measured by tissue stores (Harris, Kaley and Hickman, '44). Since the dietary intakes of caro-

TABLE 5

Relationships between plasma tocopherol (mg/100 ml) at successive stages of pregnancy and serum carotene (μg/100 ml) during pregnancy and postpartum, 1403 women

WEEKS GESTATION OF TOCOPHEROL DETERMINATIONS	AVERAGE PLASMA TOCOPHEROL LEVEL FOR GIVEN SERUM CAROTENE LEVELS (Carotene determined 4–5 weeks prior to tocopherol)						AVERAGE SERUM CAROTENE LEVEL FOR GIVEN PLASMA TOCOPHEROL LEVELS (Carotene determined 4–5 weeks prior to tocopherol)							CORRELATION COEFFICIENTS	
		< 80	80 —	160 —	≧ 240	Total		< 0.8	0.8 —	1.0 —	1.2 —	≧ 1.4	Total	Data at left	Tocopherol versus postpartum carotene
8–13 weeks	Number	24	39	9	0	72	Number	26	27	13	6	0	72	n 72	61
	Average	0.86	0.89	0.94	–	0.89 ± .02	Average	97	112	134	80	–	108 ± 5	r .141	.182
14–18 weeks	Number	32	154	34	3	223	Number	27	83	68	28	17	223	n 223	190
	Average	0.92	1.02	1.19	1.23	1.03 ± .02	Average	104	117	127	128	145	122 ± 3	r .238	.168
19–22 weeks	Number	17	147	56	8	228	Number	15	68	86	36	23	228	n 228	195
	Average	0.99	1.06	1.21	1.18	1.09 ± .02	Average	108	130	132	158	173	138 ± 3	r .332	.297
23–26 weeks	Number	19	145	72	11	247	Number	9	53	87	61	37	247	n 247	207
	Average	1.02	1.15	1.22	1.26	1.17 ± .02	Average	104	138	140	148	176	146 ± 3	r .243	.227
27–30 weeks	Number	23	126	77	19	245	Number	10	37	79	69	50	245	n 245	176
	Average	1.11	1.19	1.29	1.18	1.21 ± .02	Average	136	146	136	154	166	149 ± 4	r .170	.388
31–34 weeks	Number	23	131	66	17	237	Number	2	31	71	71	62	237	n 237	154
	Average	1.14	1.24	1.30	1.38	1.26 ± .02	Average	00	119	140	141	100	140 ± 4	r .255	.091
≧ 35 weeks	Number	15	70	51	14	150	Number	2	20	21	55	52	150	n 150	86
	Average	1.22	1.31	1.34	1.53	1.33 ± .02	Average	120	124	138	161	164	153 ± 5	r .208	.342

tene and tocopherol at their lower extremes are associated with some lowering of the blood concentrations of the respective nutrients, it might be argued that this factor of interrelation is operative here. However, the weak association noted in this study between intake of tocopherol and plasma level of the vitamin suggests that at the dietary levels encountered any such interrelationship can, at the most, play but a minor role.

The pattern of rise of plasma tocopherol during gestation was approximately linear. The first trimester level of 0.89 $\pm$ 0.02 mg/100 ml corresponds to one of 0.93 $\pm$ 0.03 mg/100 ml found in the smaller group at the 6-week postpartum visit. We therefore presume that pregnancy has not influenced the first trimester values and that they are comparable to ones from non-pregnant women. The peak rise of about 60% above these initial levels found in the large (1,575) series is high relative to the serial observations in the minor (39) series. In the latter, we have observed an average rise of 0.004 $\pm$ 0.004 mg/100 ml/week from the 30th to the 40th week of gestation, and an average fall of 41 $\pm$ 4% from levels during the last month of pregnancy to those observed at the postpartum visit. In the major series the rise from the 35th through 38th to the 39th through 42nd week was from 1.32 $\pm$ 0.02 mg/100 ml to 1.40 $\pm$ 0.05 mg/100 ml. The average value for the last period is less firmly established and the above considerations indicate that it may be slightly high. If so, 40 to 50% may be a better estimate of average increase in plasma concentration during pregnancy. The comparison with serum carotene changes during pregnancy is complicated by the association between carotene concentration and the period of entry into the obstetric clinic (tables 7 and 9, figs. 2 and 4, of Darby et al., '53b). When, as in table 5, the combined values for carotene are considered, the pattern of rise flattened out in the latter half of pregnancy, though the pattern throughout pregnancy for first-trimester entry cases is closely similar to the pattern for tocopherol. It is to be noted that

the pattern of change of serum vitamin A during pregnancy is altogether different from that of tocopherol.

It is evident that elucidation of the tocopherol-carotene interplay awaits further investigation, preferably first with repeated biochemical and dietary evaluations of groups of non-pregnant normal individuals at relatively low intakes of vitamin A.

Plasma tocopherol in relation to course and outcome of pregnancy. Plasma tocopherol levels were studied in groups of women having certain special conditions during pregnancy or postpartum. Interpretation of findings must recognize the limitations that (1) some special conditions studied occurred in but few of the total study group, (2) not all women had tocopherol determinations, (3) the single tocopherol measure, usually at 4 to 5 weeks after entry of the pregnant woman to the clinic, may not necessarily have been at an appropriate time to detect or evaluate a deviation from average level (e.g., for congenital malformations, for which the most appropriate comparison would be tocopherol determinations during the first trimester of pregnancy), (4) plasma tocopherol is not *a priori* a completely adequate measure of overall body adequacy of vitamin E nutrition, and (5) comparison of individual levels with averages for large groups fails to take account of possible individual differences in level of adequacy. Nevertheless, such findings, whether positive or negative, do bear on the search for any condition with which there may be associated an unusual level of tocopherol concentration which might allow discrimination of patients who develop a condition from those who do not.

The groups selected for comparisons of plasma tocopherol levels, and the detailed procedure of evaluation, were based on three considerations:

1. Complications of pregnancy or abnormalities of outcome which have been postulated in the literature as possibly having some association to vitamin E nutrition were investigated in detail. To this purpose, the exact tocopherol level for each affected individual was tabulated and converted to

a "standard score," the number of standard deviations from the average for the appropriate total-study group of similar age, parity, and weeks gestation at the time of the determination [i.e. (individual value minus average value of appropriate total group) ÷ standard deviation of that total group]. If the abnormality exhibited by a special group bears no relation to plasma tocopherol level, it is to be expected that the average of such individual deviations from normal level should be near zero, and the statistical significance of sampling deviation from zero can be tested approximately by a t-test. This method of analysis was adopted because of the necessity of taking into account the pattern of change during pregnancy and differences empirically assignable to age-parity.

2. Other groups with special complicating conditions were screened for relationship of condition to tocopherol-concentration levels, utilizing grouped values as coded onto punch cards at intervals of 0.2 mg/100 ml and with stage of gestation recorded in 4- or 5-week groupings. Comparisons were made within divisions by age, parity, and stage of gestation for the number of cases above, at, and below the grouping-interval containing the average for women in the total study who fell into that subgroup. These "above-at-below" distributions for special-condition cases were compared to that for the total study group, both within trimesters of gestation when the determinations were made and all together. The total study group was used for investigation of such factors as maternal and infant weight, with internal comparisons between extremes according to the above procedure.

3. If any hint of abnormality of tocopherol levels was evidenced from the exploration in (2), a more detailed examination of cases was made, either by closer evaluation of the data within the ultimate subgroups in search of source of deviation, by further discrimination within the group (such as examination of neonatal deaths by cause), or by the method of (1) above.

The results of this appraisal of tocopherol levels may be succinctly summarized: Among 7 cases of diabetes, the average tocopherol level was significantly high ($P < 0.01$). The occurrence of high tocopherol values in some diabetics has been recognized (Darby et al., '49; Bensley et al., '50). In none of the following groups of cases was there found significant deviation of maternal tocopherol levels from total average levels:

(1) Intercurrent disease conditions during pregnancy:
  tuberculosis (16 cases)
  cervicitis (12 cases)
  gonorrhea during pregnancy (10 cases)
  syphilis during or within 18 months prior to pregnancy (14 cases)
  diffuse non-toxic goiter (10 cases)
  hypertensive cardiovascular disease (5 cases)
  varicosities or hemorrhoids, or both (89 cases)
  rheumatic heart condition (9 cases)
  antepartum pyelitis, cystitis, or pyelonephritis (35 cases)
  polyhydramnios (11 cases)
  edema (186 cases)
(2) Hyperemesis (14 cases)
(3) Pre-eclampsia (76 cases)
(4) Eclampsia (9 cases)
(5) Spontaneous onset of labor (1,442 cases)
(6) Complications of labor:
  premature separation of placenta (16 cases)
  retained placenta (24 cases)
  uterine inertia (5 cases)
(7) Abortion (7 cases)
(8) Premature infants (87 cases)
(9) Congenital malformations (43 cases)
(10) Stillbirth (21 cases)
(11) Neonatal deaths (28 cases)
(12) Puerperal fever (92 cases)
(13) Mothers with history of abortion (288 cases)
(14) Mothers with history of stillbirth (48 cases)
(15) Mothers with previous livebirths now dead (126 cases)
(16) Mothers not nursing their infants on hospital discharge, classified as due to insufficient milk (51 cases)

(17) Postpartum conditions:
endometritis (64 cases)
thrombophlebitis (6 cases)
infected episiotomy or infected laceration (6 cases)
pyelitis, cystitis, or both (56 cases)

(18) Extremes in:
maternal weight
weeks gestation at delivery
length of labor
placenta weight
baby weight

Illustrative data for a few of these groups are presented in table 6.

It is obvious that changes in tocopherol nutriture as reflected in plasma tocopherol levels under the conditions of this survey are not the primary determinant in any of these complications. It is possible that the conclusions of other workers (Scrimshaw, Greer and Goodland, '49; Rauramo, '49; Bacharach, '48) which differ from our findings are a result of a different population sample, grossly different dietary intakes of the subjects, or other variation in methodology or definition. These differences are sometimes obvious in the published reports, at other times are not apparent. It is clear, however, that the therapeutic approach of Bacharach is of very different design from the present study. Hence, it is important to reemphasize that our data are from individuals *before* they developed the above-mentioned complications, and that the complications which did occur may be thought of as those in the "normal" obstetrical population such as we have described (McGanity et al., '54a). These complications were not found to be associated with evidence of lowered tocopherol nutriture.

DISCUSSION

Variations in calculated dietary intake of tocopherols from 8 to 30 mg per day during pregnancy did not influence plasma levels appreciably. When the intake was arbitrarily divided at "below 8 mg per day" this lower intake level was associated with lowered plasma values. These findings indicate

TABLE 6

Evaluation of plasma tocopherol levels in certain abnormal groups of pregnant women
Distribution of deviations of individual tocopherol values from average levels, in standardized units (see text)

UNIT NORMAL DEVIATES OF INDIVIDUAL TOCOPHEROL FROM AVERAGES	ABNORMAL CONDITIONS AND TRIMESTER OF TOCOPHEROL DETERMINATION										
	Pre-eclampsia		Eclampsia	Abortions	Stillbirths	Neonatal deaths		Diabetes	Cervicitis	Puerperal fever	
	1st–2nd	3rd				1st–2nd	3rd			1st–2nd	3rd
≧ + 2.0	1	1	0	0	1	1	0	3	1	3	2
+ 1.5 to + 2.0	2	2	1	1	0	1	1	1	1	1	2
+ 1.0 to + 1.5	4	6	0	0	3	1	0	0	1	4	5
+ 0.5 to + 1.0	2	2	0	0	3	4	0	0	4	10	7
0 to + 0.5	5	8	2	0	1	2	1	0	1	9	5
— 0.5 to 0	13	5	2	2	7	1	2	1	1	11	10
— 1.0 to — 0.5	4	5	2	3	3	3	6	2	2	7	4
— 1.5 to — 1.0	6	4	0	0	3	2	2	0	0	6	3
— 2.0 to — 1.5	2	2	2	1	0	1	0	0	1	1	1
< — 2.0	1	1	0	0	0	0	0	0	0	1	0
Total cases	40	36	9	7	21	16	12	7	12	53	39
Average deviation [1]	—.16	+.01	—.36	—.48	+.02	+.16	—.46	+1.07	+.45	+.08	+.26
± standard error	.16	.17	.33	.38 [4]	.22	.25	.29 [2]	.38 [3]	.29	.14	.16

[1] An average deviation of k unit-deviates indicates that tocopherol deviation from usual levels for the group involved is approximately 0.25 k mg/100 ml.

[2] Probability level for random deviation of this size = .11.

[3] Statistically significant at 0.01 probability level.

[4] Probability level for random deviation of this size = .21.

that when the tocopherol intake of the women is sufficiently reduced one may expect a lowering of the plasma levels. These observations, together with the demonstration that large daily supplements of tocopherol result in elevated values of the substance in the plasma (Urbach, Hickman and Harris, '52), indicate that plasma tocopherol levels may reflect intake at least in extreme situations.

Inasmuch as the dietaries of our patients were reasonably satisfactory in their content of other nutrients (Darby et al., '53b), and as there was not demonstrable health or obstetric impairment, it would seem likely that on the average the intake of tocopherol by the study group was adequate. The subgroups of women who experienced the complications of pregnancy listed did not differ appreciably from the remainder of the population with reference to the mean plasma tocopherol values. We interpret these findings to indicate that these undesirable occurrences are not manifestations of tocopherol lack in a population of this kind.

The mechanism which accounts for the association between plasma tocopherol and serum carotene and cholesterol (Darby et al., '49; Bensley et al., '50) cannot be clearly defined. A similar situation arises with the changes in plasma tocopherol which occur in disease states such as diabetes and in physiologic states in which blood lipids are altered. Recognition of this pattern emphasizes the predominant role of metabolic factors in determining the variations encountered in plasma levels of tocopherol.

SUMMARY

1. The variations in plasma tocopherol levels during pregnancy are described based upon (a) 1,575 single determinations of tocopherols and (b) a group of serial estimations on 39 women.

2. The calculated dietary intake of total tocopherol for 197 pregnant women ranged from 2.9 to 33.3 mg, with a median approximating 10 mg.

3. The average total tocopherol content of plasma increased from 0.89 ± 0.02 mg/100 ml for measurements during the first trimester of pregnancy to 1.40 ± 0.05 mg/100 ml at 39 weeks or later.

4. Increased concentration of tocopherol in plasma occurred with age and in diabetics.

5. Plasma levels of tocopherol tended to be lower in the groups with the lower calculated intakes; the differences were noted at an intake level below 8 mg of total tocopherol daily.

6. An association exists between concentrations of tocopherols and carotene in the plasma.

7. Significant deviation of maternal plasma tocopherol levels from the total average did not occur in any of the subgroups of subjects who experienced complications of pregnancy.

8. It may be concluded that no support has been obtained for the thesis that tocopherol deficiency accounts for the unexplained complications of pregnancy which are encountered in a "normal" obstetrical population as represented by our sample.

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METHODS FOR MEASURING FINGERNAIL GROWTH RATES IN NUTRITIONAL STUDIES [1, 2]

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INTRODUCTION

Information on nutritional status may be obtained from physical findings, biochemical analysis, or the composition of diets. The most conclusive evidence of a deficiency is, however, usually considered to be the alleviation of a nutritional deficiency syndrome by dietary supplementation. In nutrition surveys the curative test has been used only infrequently because most of the signs of mild nutritional deficiencies are so poorly defined that quantitative measurements cannot be made of the small changes that occur in short-term dietary studies. Long-term studies, besides being expensive, are difficult to interpret because other pertinent factors, such as variation in the basal diet, activity, season, and illness, are likely to change the conditions during the experiment.

One of the signs most frequently associated with nutritional deficiency is decrease in growth rate. This index of nutrition is, however, usually limited in its application to young, growing animals, and, in the case of the larger, slow-growing species, such as man, to long-term studies. Although growth

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of the whole body is a slow process, certain tissues, such as hair, nails and epithelium, grow at relatively rapid rates in all animals, large and small, young and old. Of these continuously growing tissues, the nails are probably best adapted to measurement, because they are readily accessible and rigid structures.

These considerations suggest that a change in the growth rate of nails induced by supplementation of the diet might provide a measure of certain nutritional deficiency states and that this measure would be applicable to mature, as well as growing, animals. Evidence in the literature suggests that the state of nutrition frequently has an effect on fingernails, but very few quantitative studies have been made of the problem because suitable methods for measuring nail growth have not been available. This paper presents a review of previous work and describes a new method for measuring fingernail growth that is suitable for short-term studies.

The medical literature contains many references to qualitative changes in fingernails thought to be associated with nutrition. These changes include transverse and longitudinal ridging, brittleness, and the "spoon nails" (koilonychia) associated with anemia. Nutrients that have been implicated are: vitamins, iron, calcium, zinc, fluorine, protein and unsaturated fats.

Although nail tissue is composed primarily of keratin, the fact that this tissue is formed by mitosis and cornification of epithelial cells indicates that the growth of nails involves much more than the synthesis of protein. Nail tissue has been shown to contain cholesterol (Hotta and Takazi, '37) and various metals (Goldblum et al., '53). Sinclair ('48) has stated that deficiencies of at least 11 nutrients probably cause alterations in the epidermis in mammals. The wide variety of nutrients that may have a role in the growth of nails presents many possible applications for nail growth studies in the field of nutrition. The numerous observations of nail anomalies associated with endocrine disorders and disease, recently reviewed by Ronchese ('51) suggest further that

quantitative nail growth studies might be of value in following the course of such metabolic derangements. As with other clinical and nutritional measures, however, the likelihood that various factors may affect the response necessitates careful control and interpretation of all studies (Bean, '50).

Quantitative studies of fingernail growth over periods of a month or more have been made by several workers. In 1930, Voit reported a long-term study on the weight of fingernail and toenail clippings of three subjects. He related the weight of the clippings to age, body growth, and hair growth, to different fingers, hands, months, and years, and to the frequency of cutting, surface area, and moisture content of the nails. Halban and Spitzer ('29) cited studies of earlier workers dating back to research by Robert Boyle in 1684. Halban and Spitzer showed that, during pregnancy, fingernail growth rates were one-fourth to one-third higher than normal. These workers, and others (Bau Kien-Tsing, '38; Wigand, '37) usually measured growth by the distance a stain on the nail advanced from the cuticle. Clark and Buxton ('38) improved this procedure by making measurements from the lunula with the aid of an illuminated magnifying glass. They showed that the nails on the longer digits grew more rapidly, but that all responded similarly to seasonal and other variations. Using the thumbnails only for further studies, they showed that growth was essentially the same for left and right thumbs, for male and female, and for ages 10 to 23 years, but that it was greater in the summer and for nail-biters, and that there was wide variation between individuals.

Using the same technique, Gilchrist and Buxton ('39) studied the relation of fingernail growth to nutritional status of East London elementary school children. They found a highly significant difference in fingernail growth rates between children rated as having "subnormal" nutrition, and those rated as "normal" or as "excellent," but no significant difference between the "normal" and "excellent" groups. They recognized the subjective errors and probable over-

lapping of their clinical ratings, but felt that the low average economic level of the "subnormal" group gave added support to the clinical assessment of that group. It is pertinent that the English school medical inspections were based on "general physical appearance" (Dunstan, '38), rather than on clinical signs associated with specific nutrient deficiencies. Since the clinical ratings may have been influenced by factors that had little effect on fingernail growth, it is not surprising that Gilchrist and Buxton found relatively small differences in the nail growth rates of their three clinically-rated groups.

Petersen ('33) has stated that meteorological conditions, particularly barometric pressure, and the period of the menstrual cycle affect the rate of fingernail growth. Further studies are needed, however, because his observations were limited to one subject, and the negative growth rates (retraction of the nail) recorded on some days throw some doubt on the accuracy of the method used (not stated) for his meaurements.

Basler ('37) noted wide variation in nail growth from hour to hour. Using a special "biomicrometer," he measured the increase in space between two brass strips, one cemented to the nail, the other cemented to the skin and overlapping the nail. The slower growth rate of nails at night observed by Basler may be caused by reduced blood pressure since Riddle ('08) has shown that the growth of feather barbules is affected by diurnal variations in blood pressure, as well as by nutrition.

Bean ('53) has recently reported a 10-year study of the growth of his left thumb nail. His method of measuring the time required for a mark on the nail to grow from the cuticle to the point of separation of the nail from its bed was not sensitive because it gave only average growth rates over periods of approximately 120 days. His measurements showed a slight but consistent decline in average growth rate with age and a retardation of growth during an attack of mumps, but they did not reveal any relationships of nail growth to season, geographic location, occupation, or other factors. Bean showed that weights of fingernail clippings are subject to

large errors because of wearing away of the nail. He also cited early observations on retarded nail growth in paralysis and immobilization. In 1871, Weir Mitchell had observed that nail growth was retarded in paralyzed hands and that growth was resumed prior to return of motor power. Head and Sherren in 1908 had observed a similar retardation of nail growth when the hand was immobilized in a splint or cast, but massage stimulated the growth.

EXPERIMENTAL PROCEDURE

Thickness or weight measurements were not considered to be satisfactory for short-term measurements of nail growth because it takes about 5 months for changes in thickness to grow out to where they can be measured (Voit, '30), and because the terminal portion of the nail may wear away rapidly (Bean, '53). Increase in the length of nails involves measuring the distance between a mark on the nail and a reference point on the finger. Since nails grow at the rate of about 0.1 mm per day, this distance is so small in short term experiments that magnification is necessary to obtain accurate readings. Measurements with a microscope are difficult because of movement of the finger (Basler, '37). It was felt that photographs would be more suitable than direct measurements on live subjects because photographs permit optical enlargement of the distance to be measured. Photographs also allow rapid collection of the data with more thorough examination of the permanent records.

The greatest problem in measuring fingernail growth is the choice of a reference point for the measurements. The cuticle, used by early workers, is inaccurate for short-term studies because it can easily be pressed back. The same applies to the point of separation of the nail from its bed. The lunula frequently is not visible on all fingers and does not appear as a sharp line when viewed through the nail with a magnifying glass. It does not show clearly on ordinary photographs, and it is said to be affected by pathologic

changes that affect the nails or nail bed (Anonymous, '34). In the studies reported here, three reference points have been tested, the phalanx bone, the skin near the nail, and the lunula.

Comparison of x-ray and skin-photographic methods

The skin between the nail and the terminal finger joint offers a possible reference point that has not previously been investigated. The reference point could be either a mark made on the skin, or the small skin wrinkles in this area. Since nutrition survey subjects might object to semipermanent skin markings, a study was made to determine whether the skin wrinkles would provide suitable reference points.

For an "absolute" standard reference point in these studies, it was decided to use the bone rather than any portion of the soft tissues. The root of the nail is closely attached to the terminal phalanx by means of dense connective tissue. To obtain growth records, the nails were marked with a material opaque to roentgen rays (a deep scratch in the nail was filled with bismuth amalgam) and an x-ray photograph was made. At the end of a 41-day growth period, another x-ray photograph was made under identical conditions. The two photographs of the bone were then exactly superimposed, and the distance the scratch-mark had advanced was measured with the aid of a microscope ocular micrometer scale and a magnifying glass. On the same days the x-ray photographs were taken, ordinary photographs (slightly larger than life-size) were also taken. These were read in a similar manner by superimposing the images of the skin wrinkles and measuring the distance the mark on the nail had advanced. In all cases, 5 individual readings were made and the averages were converted to microns per day, using factors obtained by photographing a metal caliper rule under identical conditions.

Nail growth data were obtained by the x-ray and skin-photographic methods described above from 6 subjects, rang-

ing in age from 6 to 52 years, using the index, middle, and ring fingers of each hand. To investigate the effects of different fingers, hands, and subjects, as well as the two methods used, a factorial analysis (Cochran and Cox, '50) was applied to the 72 items of data. The summary of the analysis of variance data, presented in table 1, shows that there was no significant difference between the two methods, though

TABLE 1

Analysis of variance comparison of x-ray and skin-photographic methods for measuring fingernail growth

SOURCE	DEGREES OF FREEDOM	MEAN SQUARE
Method	1	15.68 [1]
Fingers	2	153.45 [2]
Subjects	5	1,797.39 [2]
Hands	1	132.31 [2]
Error	37	13.33

Mean fingernail growth rates
Microns per day

FINGER	X-RAY	SKIN-PHOTOGRAPH
Index	103.8	101.8
Middle	107.4	107.2
Ring	106.7	106.1
Mean	106.0	105.0

[1] Not significant at the 5% level.
[2] Significant at the 1% level.

there were significant differences in the nail growth rates between fingers, between right and left hands and very large differences between subjects.

The overall averages indicated a difference of one micron between the two methods. According to the sensitivity of this statistical analysis, the odds were better than 9 to 1 that a difference of three or more microns would have been found to be significant. It is concluded from this analysis that there is virtually no bias or inaccuracy in the photo-

graphic method, using the x-ray method as an absolute reference for measuring growth.

Reproducibility of the skin-photographic method

Having established that the skin-photographic method gave a reliable measure of nail growth as compared with the x-ray method, when used with a 41-day growth period, tests were made to determine the reproducibility of the skin-photographic method when adapted to short-term (one week) studies. In short-term studies the small distance that the scratch-mark image advanced was increased, to facilitate measurements, by taking enlarged photographs (6.4 ×) of a single finger. Duplicate photographs, two at the beginning and two at the end of the growth period, were taken to provide a measure of variation caused by positioning the finger. The dispersion of nail growth measurements obtained from different combinations of these photographs gave a measure of the reproducibility of the method.

Photographs were made of the nails of the left middle fingers of 5 subjects, ranging in age from 7 to 35 years, under the following conditions. The camera was prefocused on a three-fourths inch square opening in a firmly mounted piece of sheet metal, and the finger was gently pressed upward against this frame. The illumination was standardized by clamping two 375-watt reflector-type photoflood bulbs 8 inches from the frame. The exposure (two seconds at f : 22) was controlled by an automatic timer switch connected to the lights. Two photographs were taken at the beginning of the growth period, and two photographs were made at the end of one week. Replicate growth measurements were thus obtained, one from each pair of photographs. Each growth measurement was based on 5 individual readings of the photographs, as in the previous experiment. Different skin wrinkles were used to superimpose the photographs for each reading.

The standard deviation of the growth measurements, calculated from the analysis of variance, was 6.88 μ, and the coefficient of variation was 6.3%.

Lunula-photographic method

Since the skin-photographic method did not have the desired degree of precision when used with short growth periods, tests were made to determine whether the lunula could be used as the reference point for nail growth measurements. Photographic conditions were selected to obtain maximum contrast of the lunula. It was illuminated with light of a wavelength absorbed by hemoglobin and oxyhemoglobin, and high contrast film and developer were used. The thumbnail was studied because the lunula of the thumbnail is usually more distinct than that of the other fingernails. Details of the technique follow.

The thumbnail was lightly scratched near the lunula with a sharp scalpel blade. The scratch was filled with red pigment (glass-marking crayon), and the excess was wiped off. A few drops of glycerol were applied to the nail, and the nail was *very lightly* pressed upward against a microscope slide that was firmly mounted. The glycerol served as an optical mounting medium to prevent reflections from the surface of the nail. Care was taken to avoid pressing hard against the glass because blood would be forced out of the nail-bed capillaries and the lunula would have less contrast. The camera was clamped vertically over the microscope slide and *prefocused* on a marked area of the slide. The fingernail was illuminated with two General Electric H 100 SP 4 mercury vapor spotlights, one on each side mounted at about a 45° angle. A special transformer and socket (admedium) are required for *each* bulb, and the bulbs should be turned on 5 minutes before photographs are made. Corning glass filters no. 351 and no. 978 [3] were placed in the camera optical system to isolate the 546 mμ mercury line. A Bausch and Lomb, Type II photomicrographic camera with 48 mm lens and extension bellows was used to obtain negatives enlarged about 6 times. Eastman Kodak Contrast Process Panchro-

[3] Corning now manufactures a single filter (no. 4–102) to isolate the 546 mμ mercury line.

matic sheet film and D-8 developer were used. One-second exposure at f : 22 with over-development (4 minutes instead of two minutes tray development) gave negatives of the desired density and adequate contrast. Tank development (5 minutes) has also given satisfactory results with D-8 developer.

One week later the nail was again photographed under exactly the same conditions (lens distance, bellows extension, etc.). The two developed negatives were placed against a bright light source (Keystone Overhead Projector) and the two images of the lunula were carefully superimposed. The distance between the two positions of the scratch mark was measured with a microscope ocular micrometer scale containing 140 divisions in 8.4 mm and a 7.5 × magnifying glass (Bausch and Lomb linen tester no. 81–34–46). The negatives were separated, then reread 4 more times. The average of the 5 readings was converted to microns of growth per day by using appropriate factors derived from a photograph of an accurate (caliper) scale taken under the same conditions.

Photographs were made of the left thumbnails of 9 subjects (5 male, 4 female), ranging in age from 10 to 60 years. As in the test of reproducibility of the skin-photographic method, duplicate pictures were taken at the beginning and at the end of the growth period, and the dispersion of the growth measurements was used to measure the reproducibility of the method. The standard deviation of the growth measurements, calculated from the analysis of variance, was 3.23 μ, and the coefficient of variation was 3.1%.

Lunula-transparency method

Preliminary tests revealed that it was somewhat easier to align a photographic negative with a positive transparency than with another negative. Tests were made, therefore, to determine whether the use of positive transparencies would increase the precision of the measurements. For this study,

the initial films used in the lunula photographic study were printed on Eastman Kodak Panatomic X film. These transparencies were read, as before (5 readings each) against the final negatives to obtain mean growth measurements for each of the 9 subjects. The standard deviation of these growth measurements, calculated from the analysis of variance, was 2.19 μ, and the coefficient of variation was 2.2%.

DISCUSSION

The relative precision of the short-term skin-photographic, lunula-photographic, and lunula-transparency methods is inversely proportional to the ratio of their variances, 47.3, 10.4 and 4.8, respectively. Thus, the lunula-transparency method is 2.2 times as precise as the lunula-photographic method and 9.9 times as precise as the skin-photographic method. The lower precision of the skin-photographic method is attributed to distortion of the skin by contact with the focusing frame. In the lunula methods, the increase in precision and ease of reading of transparencies probably justifies the extra work required to print them. Since the original photographs are taken in exactly the same manner, whether or not positive transparencies are used, this choice can be made after the experiment is completed.

As carried out in this laboratory (5 individual readings from each pair of films), the precision of the lunula-transparency method, when applied to an individual, would be sufficient to show a difference in growth rate greater than 5 μ per day to be significant at the 5% level. That is, with an average fingernail growth rate of approximately 100 μ per day, application of the method should reveal a difference greater than about 5% in mean growth rate.

Although the lunula may change over a period of years, or with disease (Anonymous, '34), it appears to be a relatively stable reference point for short-term studies. Slight variations in the edge of the lunula would have little effect on the readings because the negatives are placed in register

by superimposing the two images of the entire lunula, rather than by measuring from a specific point on the edge of the lunula. The greater precision of the lunula methods makes them preferable to the skin-photographic method for short-term studies. With subjects whose lunula is indistinct, however, the skin-photographic method could be applied, preferably with a growth period longer than one week to increase precision. The equipment required for the lunula-photographic method can be adapted easily to the skin-photographic method.

These methods for measuring nail growth rates in short-term studies provide a means for testing the effects of dietary supplementation, or other treatment, on nail growth rates, with each subject serving as his own control. A consistent increase in the fingernail growth rate of a subject following dietary supplementation with a nutrient would suggest that the previous nutritional status of the subject had been less than optimal with respect to that nutrient, provided, of course, that other factors affecting nail growth had remained unchanged. Since the control of extraneous variation between successive measurement periods becomes increasingly difficult as the length of the experimental period is increased, methods for measuring nail growth over short time periods are to be preferred.

Animal studies (in which a tattoo mark could be used as the reference point) are needed, as well as controlled studies with humans, to answer questions concerning the sensitivity of nail growth responses to various nutrients, drugs, metabolic disturbances, disease, and environmental factors before the place of nail growth tests in medicine, physiology, and nutrition surveys can be evaluated. Fingernail growth measurements offer an objective measure of anabolic processes that is readily obtainable without discomfort to the patient. Since the original observations are recorded permanently in photographic form, they may be checked at any time. The methods described here require little equipment and skill beyond those needed for ordinary photographic procedures.

SUMMARY

Evidence in the literature has shown that fingernail formation is probably influenced by the state of nutrition, endocrine factors, disease, and environmental factors. It is suggested that quantitative measurements of fingernail growth response following dietary supplementation might be useful in assessing the nutritional status of individuals. A method for measuring nail growth rates over short time periods is presented which was found to be capable of detecting differences in fingernail growth rates of approximately 5%. The method is based on measuring the distance a scratch-mark on the thumbnail advances with respect to the lunula during one week. Enlarged photographs are used to record the positions of the mark and increase the precision of the measurements. The method gives a permanent, objective measure of anabolic processes without discomfort to the subject.

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THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

XIV. FURTHER STUDIES OF THE EFFECT OF HYDROGENATED COCONUT OIL ON ESSENTIAL FATTY ACID DEFICIENCY IN THE RAT [1,2]

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TWO FIGURES

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The direct relationship between increasing amounts of essential fatty acids in the diet and the increase in body weight of male rats has been reported by Greenberg et al. ('50). Based on these findings, a quantitative method of assay of essential fatty acids has been successfully employed in our laboratories (Deuel et al., '51). The fact that the administration of hydrogenated coconut oil to rats on fat-low diets accentuates the essential fatty acid-deficiency symptoms has been reported by Evans and Lepkovsky ('32), by Sinclair ('36), and by Deuel et al. ('51). The depression of growth which is observed under these conditions can be completely counteracted by adequate supplementation with linoleate.

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The present results are concerned with the effects of the administration of varying levels of hydrogenated coconut oil to weanling rats receiving an otherwise fat-deficient diet, on the time of depletion, on the weight of the animal at depletion, and on the response of the animal subsequent to supplementation with linoleate. In addition, further data are available on the cholesterol concentration in the liver and plasma of rats given fat-free or hydrogenated coconut oil diets during the depletion period. Alfin-Slater and co-workers ('54) have reported that increases in liver cholesterol content seem to be associated with essential fatty acid deficiency.

EXPERIMENTAL

Series I. Ninety-nine weanling male rats and 107 weanling female rats of the U.S.C. strain were divided into 4 groups and placed on the diets 50, 51, 52, and 53, the composition of which is listed in table 1. The animals were allowed to continue on their respective diets until the typical essential fatty acid deficiency symptoms were observed, namely, a plateau in weight and the appearance of skin lesions on the tail. At this time, the average weights of the animals were calculated and the average time for depletion was observed. The results are shown in table 2.

Series II. One-hundred and ninety-nine weanling male rats of the U.S.C. strain were given a fat-free diet adequate in all other respects (diet 100, table 1); 70 weanling rats of the U.S.C. strain were placed on a similar diet to which 30% saturated coconut oil had been added (diet 101, table 1). The animals were allowed to continue on these diets until depletion, at which time they were supplemented for a period of 8 weeks with 0, 20, or 40 mg linoleate daily in the case of the animals on fat-free diets and 0, 20, 40, or 80 mg linoleate daily in the case of the animals on diet 101. The results listing the average weight of the animals at depletion and the time at which depletion was reached are shown in table 2. The effect of the administration of linoleate to these animals is recorded in table 3. Groups of animals on diet 101, subsequently given

TABLE 1

Composition of diets

DIET COMPONENT	SERIES I				SERIES II		SERIES III		
	Diet 50	Diet 51	Diet 52	Diet 53	Diet 100	Diet 101	Diet 102	Diet 103	Diet 104
	%	%	%	%	%	%	%	%	%
Casein, vitamin test [1]	20.0	20.0	20.0	20.0	20.0	28.0	...	...	...
Casein, commercial	...	...	...	...	...	...	20.0	24.0	24.0
Sucrose	72.0	67.0	57.0	42.0	72.0	32.0	72.0	53.0	53.0
Hydrogenated coconut oil	0	5.0	15.0	30.0	0	30.0	0	...	15.0
Cottonseed oil (refined, winterized)	...	...	...	...	...	...	...	15.0	...
Solka Floc [2]	4.0	4.0	4.0	4.0	4.0	5.0	4.0	4.0	4.0
Salt mixture	4.0 [3]	4.0 [3]	4.0 [3]	4.0 [3]	4.0 [4]	5.0 [4]	4.0 [4]	4.0 [4]	4.0 [4]
Fat-soluble vitamins	[5]	[5]	[5]	[5]	[6]	[6]	[6]	[6]	[6]
Water-soluble vitamins, gm/kg diet [7]	3.78	3.78	3.78	3.78	4.57	4.95	4.57	4.28	4.28
Vitamin B_{12}, μg/kg diet	50.0	50.0	50.0	50.0	50.0	67.5	50.0	60.0	60.0

[1] General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Obtained from The Brown Co., San Francisco, California.

[3] U.S.P. no. 14.

[4] L. G. Wesson, Science, *75:* 339–340 (1932).

[5] Two drops of a mixture containing 2.188 gm Napco (800,000 U.S.P. units vitamin A and 80,000 U.S.P. units vitamin D per gram) and 51.471 gm mixed tocopherols (34%) made up to 200 ml with propylene glycol.

[6] One-tenth milliliter per animal/wk. of a mixture containing 8.72 gm Nopsol (100,000 U.S.P. units vitamin A and 20,000 U.S.P. units vitamin D per gram) and 25.70 gm mixed tocopherols (34%) made up to 100 ml with propylene glycol.

[7] Water-soluble vitamins. The water-soluble vitamin mixture had the following composition: choline chloride, 52.94%; thiamine chloride hydrochloride, 0.95%; riboflavin, 1.90%; pyridoxine hydrochloride, 0.71%; calcium pantothenate, 1.77%; *p*-aminobenzoic acid, 26.47%; inositol, 13.24%; nicotinic acid, 1.60%; folic acid, 0.26%; and biotin, 0.03%. Menadione was added to the vitamin B mixture to the extent of 0.13%.

zero or 80 mg of linoleate, were sacrificed at the termination of the assay period and free and total cholesterol determinations were performed on extracts of plasma and liver by a modification of the Sperry-Schoenheimer method as reported by Nieft and Deuel ('49). The latter results are summarized in table 4.

Series III. In an attempt to correlate the increase in liver cholesterol with the symptoms of essential fatty acid deficiency usually observed, weanling male rats were divided into three groups and placed on a fat-free diet, a diet containing 15% cottonseed oil, and a diet containing 15% saturated coconut oil respectively. Ten animals per group were sacrificed after varying time intervals indicated in figures 1 and 2 and total cholesterol determinations were performed on extracts of plasma and liver as described previously.

RESULTS AND DISCUSSION

The effect of increasing levels of hydrogenated coconut oil in the diet on the length of depletion of essential fatty acids in the rat is shown in table 2. There was a steady decrease in the duration of the depletion period with the increase in concentration of hydrogenated fat in the diet, and this effect was somewhat more pronounced in males than in females. The weight of the animals at the time of depletion was also markedly affected by the presence of hydrogenated coconut oil. Although the effect seems to take place at a level of 15% in the case of the female rats, the lower weight at depletion of male rats is apparent only at a concentration of 30% (series 1). The reduced time of depletion and lowered depletion weight of male rats fed 30% hydrogenated coconut oil are confirmed by the results reported in series II where rats fed on fat-free diets were depleted of essential fatty acids in 16.6 weeks at a weight of 218.5 gm as compared with rats fed the 30% hydrogenated coconut oil which were depleted at 13.2 weeks at 167.4 gm.

The ability of rats depleted of essential fatty acids on fat-free and 30% hydrogenated fat to respond to varying amounts

of linoleate is shown in table 3. Rats on the fat-free diet supplemented with 20 mg daily of methyl linoleate gained 57.5 gm as compared with a gain of 63.3 gm exhibited by the rats fed 30% hydrogenated coconut oil and supplemented with the same amount of linoleate. Similarly, the weight gains when the linoleate was given at the level of 40 mg daily were 79.4 and 83.8 gm respectively, even though the animals fed a 30% hydrogenated coconut oil diet were depleted of essential fatty acids at a much lower weight than the rats on the fat-free diets, 167.4 gm as compared with 218.5 gm. The fact that the

TABLE 2

The effect of level of hydrogenated coconut oil in the diet on the duration of the depletion period and the body weight at depletion of rats on an essential fatty acid-free diet

CATEGORY	FAT IN DIET (HYDROGENATED COCONUT OIL)			
	0%	5%	15%	30%
Male rats				
Series I				
Diet number	50	51	52	53
Number of rats per group	22	24	26	27
Average weight at depletion,[1] gm	209 ± 4.9	204 ± 4.2	200 ± 4.7	165 ± 4.0
Average time for depletion,[1] wks.	12.9 ± 0.4	11.5 ± 0.4	10.9 ± 0.4	11.2 ± 0.3
Series II				
Diet number	100	..	..	101
Number of rats per group	199	..	..	70
Average weight at depletion,[1] gm	218.5 ± 2.2	..	..	167.4 ± 2.2
Average time for depletion,[1] wks.	16.6 ± 0.2	..	..	13.2 ± 0.3
Female rats				
Series I				
Diet number	50	51	52	53
Number of rats per group	23	28	28	28
Average weight at depletion,[1] gm	168 ± 5.2	171 ± 3.5	156 ± 3.2	138 ± 2.6
Average time for depletion,[1] wks.	12.4 ± 0.5	12.4 ± 0.4	10.8 ± 0.3	10.6 ± 0.4

[1] Including standard error of the mean.

gain in weight of both groups of animals was the same when the linoleate was given during the assay period even though the animals starting at the lower weight had the greater growth potential, corroborates the fact that the presence of

TABLE 3

The gain in weight of male rats previously depleted of essential fatty acids on diet with or without 30% of hydrogenated coconut oil, and receiving supplements of 0, 20, 40, or 80 mg of linoleate daily over 8 weeks

GROUP NO.	CATEGORY	LINOLEATE FED DAILY	NO. OF RATS	ASSAY PERIOD [1]		
				Starting weight	Final weight	Gain-in-weight
		mg		*gm*	*gm*	*gm*
II, 1	Fat-free diet	0	12	220.4 ± 9.4	220.8 ± 12.4 [2]	+ 0.4 ± 4.1
2	Fat-free diet	20	12	215.3 ± 7.2	272.8 ± 5.2	57.5 ± 4.8
3	Fat-free diet	40	11	204.7 ± 7.7	284.1 ± 5.5	79.4 ± 3.6
II, 5	30% — Hydrogenated coconut oil diet	0	12	159.8 ± 6.1	149.5 ± 4.5 [2]	− 16.1 ± 3.5
6	30% — Hydrogenated coconut oil diet	20	12	166.8 ± 7.5	230.1 ± 8.1	63.3 ± 3.6
7	30% — Hydrogenated coconut oil diet	40	12	158.0 ± 5.8	241.8 ± 7.0	83.8 ± 4.8
8	30% — Hydrogenated coconut oil diet	80	12	152.8 ± 4.9	270.4 ± 7.9	117.6 ± 5.9

[1] Including the standard error of mean.
[2] Only 10 rats.

the hydrogenated coconut oil in the diet decreases the response of the animal to linoleate supplements.

In a previous investigation in this laboratory (Alfin-Slater et al., '54) it was observed that the rats fed a diet low in fat over prolonged periods of time developed a typical "cholesterol-type" fatty liver in which there was an accumulation of cholesterol esters in this organ. This was accompanied

by an increase in total lipid in the liver. On the other hand, plasma cholesterol levels were sharply reduced. In figures 1 and 2, it can be seen that the increase in liver cholesterol on the fat-deficient diet and the diet containing saturated

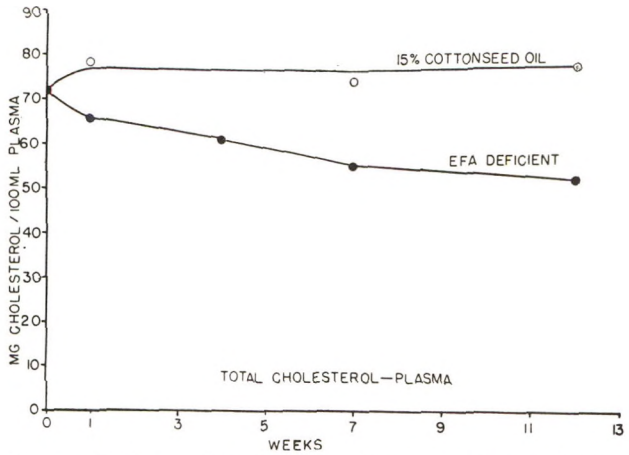

Fig. 1 The total plasma cholesterol in milligrams percent of weanling male rats receiving a diet deficient in essential fatty acids (lower curve) or one containing 15% refined cottonseed oil (upper curve) over a 12-week period.

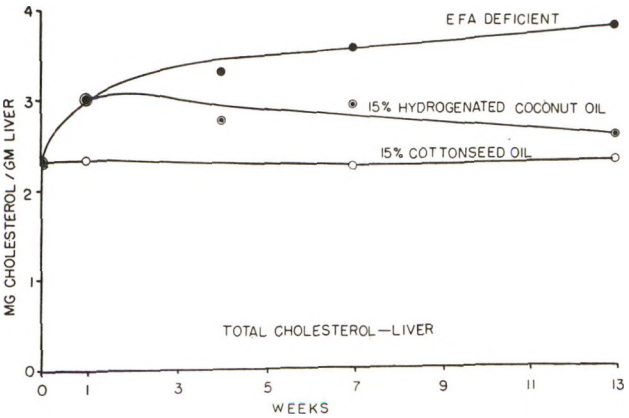

Fig. 2 The total cholesterol in the liver of rats in milligrams per gram. The rats had received from weaning a diet deficient in essential fatty acids (upper curve), one deficient in essential fatty acids but containing hydrogenated coconut oil (middle curve), or one containing 15% refined cottonseed oil (lower curve) over 13 weeks.

fat is evidenced as early as one week after the animals are placed on the respective diets. The decrease in plasma cholesterol in the fat-deficient rats is equally as marked in the one-week period. Thereafter there is a more gradual increase in liver cholesterol and a more gradual decrease in plasma cholesterol in the animals receiving the fat-low diets over the experimental period.

The animals on the 15% coconut oil diet, however, after the initial rise in liver cholesterol content, showed a gradual decrease in cholesterol concentration approaching the normal

TABLE 4

The cholesterol content of liver and plasma of rats depleted of essential fatty acids on a 30% hydrogenated coconut oil diet and subsequently supplemented with 0 or 100 mg linoleate daily for 8 weeks as compared with a control, non-depleted group

DIET NO.	LINOLEATE SUPPLEMENT	CHOLESTEROL DETERMINATIONS [1]					
		Liver			Plasma		
		Total	Free	Free	Total	Free	Free
		mg/gm	*mg/gm*	*%*	*mg %*	*mg %*	*%*
101	—	2.57 ± 0.18	1.70 ± 0.03	66.2	50.3 ± 3.0	12.5 ± 1.1	24.8
101	+	2.20 ± 0.06	1.64 ± 0.06	74.5	49.6 ± 2.8	13.9 ± 0.87	28.0
103 (Control)	—	1.99 ± 0.12	1.73 ± 0.07	86.9	58.1 ± 6.0	..	..

[1] Including standard error of mean.

value at the end of the 13-week experimental period. The partial alleviation of this condition by the presence of hydrogenated coconut oil in the diet may be due to the possibility that the short-chain fatty acids contained in the hydrogenated fat can form the necessary esters with cholesterol essential for establishing a normal equilibrium. In experiments in which rats were depleted of essential fatty acids on a 30% hydrogenated coconut oil diet and then subsequently supplemented with either zero or 100 mg linoleate daily, it can be seen in table 4 that although the plasma cholesterol levels are practically identical in the two experimental cases, a further

decrease in cholesterol esters in the liver approaching the normal control value obtains when the essential fatty acid, linoleic, is added to the diet.

SUMMARY

The effect of the addition of hydrogenated coconut oil to the otherwise fat-free diets of rats has resulted in the depletion of essential fatty acids in a shorter time interval and at a lower animal weight than when the rats were fed a fat-free diet alone. However, the continued presence of hydrogenated coconut oil in the diet had no *apparent* inhibitory effect on the subsequent response of the animal to linoleate although the animals depleted with hydrogenated coconut oil in the diet had a greater growth potential than the animals depleted on the fat-free diet.

In rats receiving diets deficient in essential fatty acids, there was an increased cholesterol concentration in the liver and a decreased cholesterol content in the plasma after one week. However, although the condition was further aggravated thereafter in the animals on the fat-free diets, cholesterol levels of the rats receiving hydrogenated coconut oil in the diets gradually returned to normal. This effect may be due to the availability of short-chain fatty acids contained in the hydrogenated fat for esterification of cholesterol.

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UTILIZABILITY OF FLUORINE FOR STORAGE IN THE RAT WHEN ADMINISTERED IN MILK [1]

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The continued search for a vehicle to carry inorganic fluorides to that portion of the population not served by communal fluoridation has led frequently to the suggested use of milk. Milk seems to be a fairly logical food for this purpose, since it is consumed by a large percentage of the adolescent population. Many workers have criticized such a use of milk on the grounds that the added fluoride would not be available for metabolic purposes. However, little actual experimental evidence exists to support this assumption, although previous workers have considered a number of the basic problems pertaining to fluorine retention (Roholm, '37; McClure, '46; Muhler, Hine and Day, '54). It has been shown by Evans and Phillips ('39) that the naturally occurring fluorine of cow's milk can be partially utilized as evidenced by skeletal storage of the element. However, this is to a limited degree, as indicated by the authors' statement that, "It appears that the fluorine naturally occurring in milk is less readily available for storage in the skeleton." Weddle and Muhler ('54a) have reported that when calcium and magnesium are added to a sodium fluoride solution, in the same ratio in which they are found in milk, less fluorine is stored in the skeleton than in the absence of these elements. However, since no data were available in which milk itself was

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used, it was considered of importance to investigate the storage of fluorine in the skeleton of the rat when identical amounts of fluorine were given in milk and in aqueous solution.

EXPERIMENTAL

A total of 140 rats of the Sprague-Dawley strain were divided into three series for use in these experiments. Series I consisted of three groups of weanling rats which received 5 p.p.m. of fluorine either in their dry diet, in milk or in drinking water. Series II was composed of animals 55 to 60 days of age and those received 5 p.p.m. of fluorine in either milk or drinking water, while in series III a group of weanling rats received 2 p.p.m. of fluorine in milk or water. Following the consumption of the fluoridized supplements, all groups received the same low-fluorine stock corn diet and fluorine-free drinking water.

Since the criterion of utilization of the fluorine was skeletal storage, it was necessary to control carefully the amount of fluorine received by the rats of each group in a particular series. This was accomplished in the following manner. All animals were housed in individual cages and each day a known amount of either dry diet, milk or water was given to each rat in order to furnish the fluoride supplement. If every rat consumed the entire amount, the following day the amount was increased by one gram (or milliliter). This procedure was followed until one rat in the group failed to eat the entire amount. When this occurred, the amount not eaten was subtracted from the food given and suitable allowances were made for other rats in the series. The fluoride supplements were made available to all of the rats in the early morning, and after each rat in the particular series had consumed all of the supplement, fluoride-low drinking water ($F = 0.06\ \mu g/ml$) and a fluoride-low stock corn diet ($F = 0.5\ \mu g/gm$) were provided. By following this procedure accurately, it was possible to supply all animals in a particular series with almost identical amounts of fluorine. No attempt was made to determine the total amount of basal diet consumed by the in-

dividual animals, since the amount of fluorine available from this source was too small (Weddle and Muhler, '54b) to be a factor in these experiments.

The animals were housed in raised-bottom screen cages in an air conditioned room. The duration of the experiment was 40 days for each series, after which time the animals were sacrificed by ether and the right femur from each animal

TABLE 1

Storage of fluorine in the rat when supplied (a) as part of the dry food, (b) in a fluid milk supplement and (c) in drinking water

FLUORINE CARRIER	TOTAL FLUORINE INGESTED	FLUORINE IN FEMUR		TOTAL FLUORINE IN WHOLE CARCASS	FLUORINE RETAINED IN WHOLE SKELETON
		Conc.	Total		
	mg	*P.P.M.*	*μg*	*μg*	*%*
Series I, F conc. 5 p.p.m.					
Dry food	1.64	264	33 ± 0.24 [1]	825 ± 75	50.3
Milk	1.64	272	39 ± 1.00	952 ± 30	58.1
Water	1.64	327	45 ± 1.70	1029 ± 119	62.6
Control		34	4 ± 0.01	135 ± 12	...
Series II, F conc. 10 p.p.m.					
Milk	6.87	957	147 ± 17	3868 ± 570	55.4
Water	6.87	1040	175 ± 213	4135 ± 379	60.2
Series III, F conc. 2 p.p.m.					
Milk	1.33	99	17 ± 4	396 ± 68	29.8
Water	1.33	183	31 ± 4	719 ± 53	54.0

[1] Standard deviation.

was removed for fluorine determination. The procedure for fluorine analysis (Muhler, Nebergall and Day, '54) and preparation of the rat for total carcass analysis have been described previously (Weddle and Muhler, '54a).

RESULTS AND DISCUSSION

The combined data for the three experimental series are found in table 1. The data obtained from the animals in series I indicate that the fluorine supplied in the dry diet is less avail-

able than the same amount of fluorine either in the milk or in aqueous solution. Both the total amount of fluorine in the femur and in the whole animal suggest this conclusion. As a result of previous work it was not expected that there would be no differences in the skeletal storage of fluorine when the fluorine was fed either in milk or in aqueous solution. However, when the total amount of fluorine ingested is compared to the total amount found in the whole animal (column 6, table 1) there is seen some indication that more flourine may be available when it is supplied in the drinking water; these differences are so slight however, that these are considered to be not truly significant.

The series II experiment was designed to explain why such slight differences were found in the storage of fluorine in these two groups of animals. In previous experiments, using calcium and magnesium salts in the presence of fluorine, Weddle and Muhler ('54a) found differences in fluorine storage. Several explanations may be offered for these divergent results. The age of the animal, the concentration of fluorine used in the milk experiment or the possibility that some factor or factors present in milk, but absent in the diets in which the inorganic salts were used, decreased the utilization of the fluorine. From a consideration of these factors, it was decided to double the fluoride concentration in an attempt to decrease the ratio between the suggested interfering ions (calcium and magnesium) and fluorine, and to use older animals on the assumption that there would be a decreased ability to store fluorine. However, the data obtained in the series II experiment indicate that when 10 p.p.m. of fluorine is added to milk no real difference in the amount of fluorine stored in the skeleton is found as compared to the results of a similar group receiving the same amount of fluorine in aqueous solution.

The last experiment, series III, was designed to determine the availability of fluorine when added to milk or water at a concentration similar to that which could be added to milk as a fluoride supplement. If 1.0 to 1.5 mg of fluorine a day is

considered optimum for dental health, the amount received by the animals in series III (2.0 p.p.m.) would be within this range. Futhermore, this level was considered more practical from an analytical standpoint than one-half this concentration. The results of this experiment indicate a significant difference between the availability of the element in milk and in water. These data confirm previous work (Weddle and Muhler, '54a) and indicate that when an amount of fluorine similar to that suggested for fluoridation of a community water supply is used to fortify milk, 45% less fluorine is stored in the whole animal than when an identical amount of fluorine is placed in water. These latter data provide evidence that fluorine in concentrations suitable for routine human consumption cannot be placed in milk and still retain a satisfactory degree of metabolic availability.

In order to confirm the latter observation another experiment was performed in which the same concentration of fluorine, as sodium fluoride, was added to mineralized milk [1 mg iron as $Fe_2(SO_4)_3$, 0.1 mg copper as $CuSO_4$ and 0.1 mg mangenese as $MnSO_4$ per 100 ml] and to water. These fluoride supplements were administered ad libitum and provided further evidence that less fluorine is stored in the skeleton in the animals of the milk group than in those receiving the fluoride in aqueous solution.

The explanation of the failure of higher concentrations of fluorine in milk to produce significant differences in skeletal storage in comparison with that obtained with fluoridated drinking water is not readily apparent. However, experience in these and other unpublished experiments indicate that the total amount of milk ingested and the level of fluorine in the milk are important considerations. Evidently, when the fluoride concentration in milk reaches 10 μg there is sufficient fluorine present to make the interfering effect of calcium and magnesium of little consequence.

CONCLUSIONS

Fluorine in the form of sodium fluoride has been shown to be less available for storage in the skeleton of the rat when

placed in milk than when fed as fluoridized drinking water. These data suggest that fluoridized milk may not be a satisfactory food supplement to use for providing fluorine to that portion of the population not served by a fluoridized drinking water supply. The interfering effect of milk on fluorine availability is decreased as the concentration of the fluorine in the milk is increased.

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