Lysine Toxicity in the Chick[1]

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That a dietary excess of most of the common amino acids, when fed to experimental animals receiving either complete diets or diets lacking an essential nutrient, causes growth retardation is well documented (Harper et al., '55), although a satisfactory explanation for this observation has not been proposed. L-lysine has been one of the amino acids most frequently studied as a supplement for dietary proteins, and both adverse and beneficial effects have been observed. Levels of lysine up to 4%, when added to adequate and limiting protein rations, caused a severe growth depression when fed to day-old chicks, which was partially overcome by added gelatin (Anderson and Combs, '52); also growth depression and toxicity symptoms were observed by Jones.[3] These symptoms are not as readily observed in older chicks (Hsu and Combs, '52) or in rats (Harris and Burress, '59) fed comparable levels of the amino acid added to complete or low protein diets. Thus, the day-old chick appeared to be the preferred experimental animal for lysine toxicity studies.

Since numerous workers (Eckel et al., '58; Iacobellis et al., '56; Gershoff et al., '59) have shown a relationship between the distribution of basic amino acids and electrolytes in rodent tissues, this study was initiated to compare the distribution pattern of lysine, Na and K in tissues of the young chick fed excessive amounts of lysine with that of the normal young chick.

Data were obtained in two experiments after supplying diets for various time intervals, to determine whether the change in electrolyte composition of the tissues as demonstrated for other species (Dickerson and Widdowson, '60; Darrow and Hellerstein, '58) would be apparent by three weeks of age in the chick.

EXPERIMENTAL

Day-old Cornish × White Rock crossbred chicks, obtained commercially, of both sexes were housed in electrically heated batteries with raised screen floors. Ten to 12 chicks were allotted at random to each pen using at least 4 pens per treatment. All of the diets were offered ad libitum and contained the following (in per cent): casein, 18; gelatin, 10; salts 5 (Briggs et al., '43), 6; corn oil, 4; glycerol, 1; DL-methionine, 0.3; choline chloride, 0.2; inositol, 0.1; and vitamins (in mg per 100 gm of ration) as follows: thiamine·HCl, 0.6; riboflavin, 0.9; niacin, 5; Ca pantothenate, 2.0; pyridoxine, 0.8; biotin, 0.02; folic acid, 0.4; α-tocopherol, 0.3; vitamin B_{12}, 0.003; and menadione, 0.05. Each week 240 ICU of vitamin D_3 and 2400 USP units of vitamin A were given orally. Sucrose was added to make each diet up to 100% and all supplements were included in the basal ration at the expense of sucrose. The percentage composition of the basal ration was: lysine, 1.73; K, 0.74; and Na, 0.40 by calculation.

Blood obtained by heart puncture, with the chicks under ether anesthesia, was heparinized for whole blood determinations and centrifuged immediately for plasma determinations. Different birds of the same groups were sacrificed by ether anesthesia for the tissue analyses since loss of blood has been implicated in altered tissue electrolyte concentrations (Widdowson and Southgate, '59). Lysine was determined

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[3] Jones, J. D. 1958 Studies on improved nutrition due to antibiotics. Ph.D. Thesis, University of Wisconsin, Madison.

by a microbiological method (Steele et al., '49) modified by the inclusion of 125 μg of hydroxylysine per tube[4] on ether extracted trichloroacetic acid preparations of serum and homogenized tissue. Concentrations of tissue components were calculated using a tissue water content of 77%. Determined tissue water was constant among treatments. Sodium and potassium were determined by flame photometry using a Beckman model B with flame attachment. Tissues were digested with fuming nitric acid and diluted to not more than 3 gm of tissue to 100 ml of water. At this dilution phosphorus at up to 4 times the maximum level found in chick tissues was observed to have no effect on the concentration of either sodium or potassium. Ninhydrin-reacting material (NRM), determined colorimetrically, was used as a measure of nonprotein nitrogen present in the tissues.

[4] Sauberlich, H. E. 1957 Relationship of hydroxylysine in microbiological assays for lysine. Federation Proc., *16*: 399 (abstract).

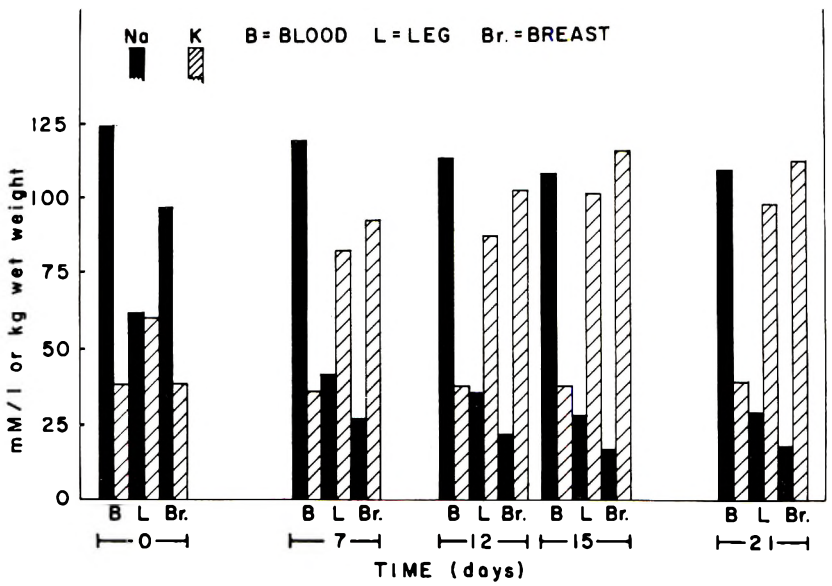

Fig. 1 The effect of age on Na and K content of selected chick tissues.

TABLE 1

The effect of 2% of L-*lysine on tissue electrolytes of young chicks*

Treatment		14 days			21 days		
		Na[1]	K[1]	Δ[2]	Na	K	Δ
		mmoles/kg	*mmoles/kg*	*gm*	*mmoles/kg*	*mmoles/kg*	*gm*
Control	Leg	41.2[3]	95.0[4]		35.5	91.5	
	Breast	23.4[4]	107.8		22.3	110.1	
	Brain				58.7	95.7	
	Heart				58.9	75.5	
				106[4]			164[4]
2% L-Lysine	Leg	49.3	81.7		46.2	93.3	
	Breast	36.2	105.6		30.9	106.7	
	Brain				57.2	95.5	
	Heart				55.9	77.2	
				47			54

[1] Values are based on fresh weight and are an average of 10 birds.
[2] Δ is the body weight change of sacrificed birds.
[3] Differences between treatments statistically significant (P = 0.05).
[4] Differences between treatments statistically significant (P = 0.01).

All values are given on a wet-weight basis and were subjected to analyses of variance as described in Snedecor ('56).

RESULTS

As in previous studies the addition of 2% of L-lysine, as the monohydrochloride,[5] to the basal ration depressed growth (table 1) and produced toxicity symptoms such as hyperirritability, nervousness, leg tremors and later leg weakness. Leg and breast Na were increased by the dietary lysine while leg K was reduced by the same treatment after the diet had been fed for 14 days. The two tissues did not react in a like manner to the dietary treatment at 14 days and only Na distribution still appeared to be affected at 21 days indicating an adaptation to the diet although growth had not resumed. Plasma Na and K showed no deviations due to treatment or age, and brain and heart tissues showed no differences in either Na or K concentrations due to treatment.

Since the change in electrolytes caused by excessive dietary lysine appeared to be a function of the age of the animal, the distribution of Na and K was determined at varying intervals in a series of chicks from one- to 22-days old (figure 1 and control values, table 2). Whole blood showed a change only in Na concentration, from 124 to 109 mmoles per liter, while the K content remained constant. Plasma values remained constant for Na and K. In control animals a relatively high concentration of Na and low concentration of K was observed in both leg and breast muscle of the day-old birds which had changed by 12 days to values more similar to those observed in older birds.

No correlation of weight gain could be made with the change in tissue electrolyte concentrations within a treatment, the change being age-dependent only.

The other experimental variables in this experiment were the addition of equimolar levels of lysine as the free base[6] and the monohydrochloride (table 2). The change in Na and K content of the tissues due to

TABLE 2

The effect of age and form of L-lysine supplement on growth and tissue electrolytes

Treatment	Tissue	7 days			12 days			15 days			21 days		
		Na[1]	K[1]	Δ[2]	Na	K	Δ	Na	K	Δ	Na	K	Δ
		mmoles /kg	*mmoles /kg*	*gm*	*mmoles /kg*	*mmoles /kg*	*gm*	*mmoles /kg*	*mmoles /kg*	*gm*	*mmoles /kg*	*mmoles /kg*	*gm*
1. Control	Leg	41.1[3]	82.3		35.7[4]	87.5[4]		27.8[4]	101.5[4]		28.8[4]	97.4[4]	
	Breast	27.0[3]	92.3		21.8[4]	102.3		17.0[4]	115.4[4]		18.0	111.9[4]	
	Body weight			64[4]			142[4]			189[4]			265[4]
2. Lysine, 2% (as HCl)	Leg	45.3	80.1		45.7[5]	79.3		35.2	89.1		38.1[5]	80.8[5]	
	Breast	32.7	90.1		29.2	98.8		21.8	103.4		21.2	102.6	
	Body weight			39			63			87			88[5]
3. Lysine, 2% (as free base)	Leg	44.4	78.5		39.7	79.4		35.0	88.7		29.5	91.8	
	Breast	31.0	87.0		26.8	101.4		21.9	109.0		18.8	102.6	
	Body weight			39			91			100			199

[1] Values are based on fresh weight.
[2] Δ is the total change in body weight for the experimental period of the birds sacrificed at that time interval.
[3] Significant difference 1 vs. 2 and 3 at 5% level.
[4] Significant difference 1 vs. 2 and 3 at 1% level.
[5] Significant difference 2 vs. 3 at 1% level.

[5] L-Lysine monohydrochloride was kindly supplied by the E. I. du Pont de Nemours and Co., Wilmington, Delaware.

[6] Obtained from Nutritional Biochemicals Corporation, Cleveland.

added lysine was more pronounced than in the first experiment and was greatest at about 12 days with the leg muscle being the tissue affected to the greatest extent. Again plasma Na and K were unaffected by treatment or age. No difference was observed either in the electrolyte pattern or growth depression between the two forms of lysine until 21 days of age at which time the birds receiving the free lysine appeared to be recovering. One might thus conclude that the effects noted are not due to an acid-base disturbance caused by the accompanying hydrochloride but are due to the lysine.

Since one effect of excessive dietary lysine appeared to be measurable changes in tissue components and since folic acid has been shown to eliminate visible symptoms of glycine toxicity in chicks without any change in plasma glycine level or metabolites of glycine (Naber et al., '56), excess folic acid was added as a treatment variable in the next experiment. No consistent difference in distribution of Na, K, lysine or NRM (ninhydrin reacting material) was observed due to added folic acid (table 3). The added folic acid tended, however, to reduce the variability between samples of a given treatment. Growth rate was again depressed by both 1.5 and 2% of lysine as evidenced by a growth depression of 48 and 62 gm after the chicks had received the diets 14 days, due to the two levels of lysine, respectively. Leg and breast muscle showed the characteristic changes in Na and K concentrations and the concentration of lysine and NRM were

TABLE 3

Tissue composition of chicks fed high levels of L-*lysine·HCl as affected by excess folic acid*[1]

Treatment	Δ	F/G[2]		mmoles/l or kg tissue wet weight			
				Na	K	Lysine	NRM[3]
	gm						
1. Control	135[4]	1.37	Leg	30.3[4]	90.8[4]	3.61[4]	44.9[4]
			Breast	16.4[4]	104.9[5]	1.14[4]	33.9[4]
			Liver	55.2	70.4	0.86[4]	35.7[4]
			Plasma	131	4.9	0.64[4]	7.3[4]
2. Lysine, 1.5%	86[6]	1.71	Leg	35.2	87.4[6]	7.65	47.0[6]
			Breast	21.9	103.6	4.81	35.9[6]
			Liver	47.3	66.7	2.34[6]	39.2
			Plasma	131	5.5	1.84[6]	8.4
3. Lysine, 2%	71	1.92	Leg	35.6[7]	79.8	11.43	55.2
			Breast	24.6	103.5	8.07	48.0
			Liver	51.7	66.8	3.81[7]	40.7[7]
			Plasma	127	5.4	2.39[7]	8.9
4. Lysine, 2% and folic acid, 24 mg/kg diet	64	1.79	Leg	41.8	81.4	9.12	56.7
			Breast	23.1	103.6	9.37	43.1
			Liver	58.0	68.1	3.01	35.4
			Plasma	132	4.9	2.04	8.9
5. Folic acid, 24 mg/kg diet	139	1.41	Leg	31.7	93.3	4.01	47.0
			Breast	17.3	104.6	1.52	37.7
			Liver	51.7	66.6	0.85	30.6
			Plasma	132	4.8	0.59	7.3

[1] All values were obtained from chicks fed the diets 14 days, and are the average of 4 pooled samples of 5 birds.

[2] F/G is grams of feed consumed per gram of gain in body weight.

[3] NRM (ninhydrin-reacting materials) expressed as leucine equivalents.

[4] Analysis of variance indicates significant difference at 1% level between treatments 1 and 5 vs. 2, 3 and 4.

[5] Analysis of variance indicates significant difference at 5% level only, between treatments 1 and 5 vs. 2, 3 and 4.

[6] Analysis of variance indicates significant difference at 1% level between treatment 2 vs. 3 and 4.

[7] Analysis of variance indicates significant difference at 5% level only, between treatment 3 vs. 4.

markedly influenced by the level of dietary lysine. This was also true for the lysine and NRM in the liver and plasma while the electrolyte concentrations were not affected by treatment in these tissues. It would thus appear that the increased lysine concentration did not cause the loss of any great quantity of non-protein nitrogen from the tissues.

DISCUSSION

Observations showed that 1.5 to 2% of L-lysine added to an adequate protein diet is toxic when fed to day-old chicks. Although these experiments yielded additional information on the toxicity syndrome exhibited by this animal, it was not possible to determine whether the changed electrolyte-amino acid patterns due to excessive dietary lysine were the cause or result of depressed growth rates. The leg symptoms seen in these birds are probably caused by the abnormal tissue composition. It is very possible that differences in tissues other than muscle which would be of physiological importance are too small to be measured by the techniques used here. It is not unexpected that all tissues did not respond in a like manner since it has been previously noted that electrolyte patterns of various tissues are affected to varying degrees in K deficiency (Eckel et al., '58) and that muscle and liver do not concentrate supplemented amino acids to the same extent (Christensen et al., '48).

Use of older animals may be the reason that similar distribution changes in the electrolyte content of tissues from animals fed rations varying in lysine and K content were not observed by Gershoff et al. ('59); and may also explain why Eckel et al. ('58) observed a tenfold increase in lysine but no change in K content of muscle in rats receiving 11.6% of dietary lysine HCl.

It is unlikely that an increased plasma concentration of lysine *per se* would be the cause of the symptoms observed here since it has been demonstrated that glycine toxicity could be eliminated without changing the levels of plasma glycine (Naber et al., '56.) The hydrochloride present in the commonly used form of lysine is not the primary cause of the toxicity. The results noted are not interpreted to be caused by starvation since the chicks continued to gain in body weight but at a slower rate; that is, it may be that the changed electrolyte pattern is observed in the chick and not in other species because the chick continues to consume the harmful diet.

That a change in the electrolyte pattern of tissues occurs during the first three weeks of life is a likely explanation for differences in susceptibility between different-aged animals and may be the reason that the young animal is more susceptible to toxicity caused by excess lysine (Anderson and Combs, '52; Russell et al., '52) and for lysine being relatively nontoxic in more "physiologically mature" animals (Eckel et al., '58; Harris and Burress, '59). Although data have been presented by Eckel et al. ('58) to show that no competition exists between K and lysine in the tissues of the rat, one interpretation of the data presented here may be that the presence of excessive lysine in the tissue prevents the development of a normal electrolyte pattern.

SUMMARY

An excess of dietary L-lysine either as the hydrochloride or free base depressed the growth rate and produced toxicity symptoms in 14- to 21-day-old chicks fed adequate protein diets.

The effect of excessive dietary lysine on tissue composition was to decrease the K concentration of muscle, increase the Na and lysine content of muscle and to increase the lysine content of the liver and plasma. No change occurred in the plasma Na or K.

A change with age in electrolyte composition of selected tissues of the chick was demonstrated. These observations suggest that the physiological immaturity of the tissue makes the very young chick susceptible to lysine toxicity.

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Utilization of Amino Acids from Foods by the Rat

V. EFFECTS OF HEAT TREATMENT ON LYSINE IN MEAT[1,2,3]

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Attempts to evaluate the nutritional quality of protein have shown that discrepancies sometimes occur between methods based upon the amino acid content of the protein and methods involving physiological criteria such as the growth performance of animals which have been fed the protein. Kligler and Krehl ('50) found that zein did not support optimum growth in young rats although it was supplemented with the essential amino acids to theoretically adequate levels. They concluded that the decreased growth rate was due to impaired digestion of zein so that all of the amino acids essential for protein synthesis were not made available to the animal within the time interval necessary for optimal utilization.

Deshpande et al. ('57) stated that "as far as cereal proteins are concerned, the presence of an amino acid in the protein, although in sufficient quantity, does not adequately determine its nutritional quality." These authors found a twofold increase in the availability of isoleucine for growth of weanling rats from an acid hydrolysate of zein over that of unhydrolyzed zein.

Observations *in vitro* have shown that the rates of enzymatic release of lysine from proteins were decreased when the proteins were dry-heated (Eldred and Rodney, '46; Pader et al., '48) or autoclaved (Hankes et al., '48), possibly as a result of chemical changes involving the ε-amino group of lysine. Gupta et al. ('58) have reported that the lysine of roller-dried skim milk was less available than that of a spray-dried preparation but Guthneck et al. ('53) found essentially no decrease in lysine availability in cooked ham, lamb or beef as compared to their uncooked counterparts. In previous studies in our laboratories, no consistent changes in the utilization of methionine (Schweigert and Guthneck, '54) or tryptophan from cooked meat proteins (Lushbough et al., '57) were observed.

The present studies were undertaken to extend the examination of the effect of heating upon the availability of lysine from meat. The rate of body weight gain in the male weanling rat was used as the criterion for evaluating the utilization of lysine as compared with the microbiologically-determined lysine from unheated and heat processed meat samples.

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[2] Two preliminary reports, "Studies on Amino Acid Availability from Meat Proteins Subjected to Varying Degrees of Heat Treatment" by C. H. Lushbough, B. S. Heller, M. R. Chutkow and B. S. Schweigert and "Effect of Heat Treatment on Availability of Lysine from Meat" by C. H. Lushbough, M. R. Chutkow, B. S. Heller and B. S. Schweigert were presented at the Symposium on Protein and Amino Acid Supplementation, Division of Agricultural and Food Chemistry, American Chemical Society Meetings, Atlantic City, 1959 and the 43rd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., 1959, respectively.

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EXPERIMENTAL AND RESULTS

Paired, fresh, uncured ham and lamb leg samples were prepared by roasting one of each pair at an oven temperature of 300°F to internal temperatures of 185° and 170°F, respectively. The roasts were then divided into two approximately equal parts, representing the inner and outer portions of the cut. Other raw samples from these meat cuts were autoclaved at 250°F for 16 hours. Paired cuts of beef round were roasted at oven temperatures of 200°, 300° or 400°F to an internal temperature of 140°F and then divided into inner and outer portions. In addition, raw cuts of beef round were cooked in an electronic oven to an ultimate internal temperature of 136°F or in an autoclave at 250°F at 15 pounds pressure for 4 or 16 hours. Samples of beef from the same carcass were ground, 4% of NaCl added and, with the cooperation of one of the meat packing companies, were canned and processed in steam-heated retorts at 240°F to F_0 values of 2, 6 or 10. (An F_0 value of 1 is equal to maintaining the temperature at the center of the can at 250°F for one minute). Detailed information on sample preparation, cooking yields and thiamine retention has been published elsewhere (Lushbough et al., '60).

TABLE 1

Composition of lysine-deficient basal ration

Ingredient	Amount
	%
Sucrose	57.7
Sesame meal	18.3
Amino acid mix[1]	10.0
Vitamin mix[2]	5.0
Salts IV[3]	4.0
Corn oil	4.7
Fish liver oil[4]	0.3

[1] The amino acid mix supplied per 100 gm of ration (in milligrams) L-cystine, 300; L-histidine·HCl, 285; DL-isoleucine, 635; L-leucine, 500; DL-methionine, 350; DL-threonine, 340; DL-tryptophan, 150; L-tyrosine, 320; DL-valine, 670; and 6.44 gm of sucrose.

[2] The vitamin mix supplied per 100 gm of ration (in milligrams) thiamine·HCl, 0.6; riboflavin, 0.6; niacin, 2; pyridoxine·HCl, 0.6; Ca pantothenate, 4; folic acid, 0.2; menadione, 3; *p* aminobenzoic acid, 30; and (in grams) choline chloride, 0.15; inositol, 0.1; sucrose, 4.71; and cyanocobalamin, 2 μg; biotin, 1 μg.

[3] Hegsted et al. ('41).

[4] Nopco XX: 2,250 USP units vitamin A and 300 USP units vitamin D/gm.

Each of the above samples was lyophilized, ether-extracted and ground to a homogeneous powder in a hammer mill. Lysine was determined microbiologically using the method of Schweigert et al. ('49).

Male weanling rats[6] were fed a commercial laboratory diet[7] for one day, weighed, and then arranged into groups of 7 each so that the average weights of the groups differed by no more than 2%. The animals were placed in individual cages with raised screen floors and fed the test diets ad libitum for 14 days. Temperature was maintained at 78°F at 75% relative humidity.

The composition of the basal ration used in these studies is given in table 1. This ration was designed to provide adequate amounts of all known required nutrients except lysine. L-Lysine or the test samples were substituted at the expense of sucrose in the basal ration. The average 14-day weight gains of animals fed the various

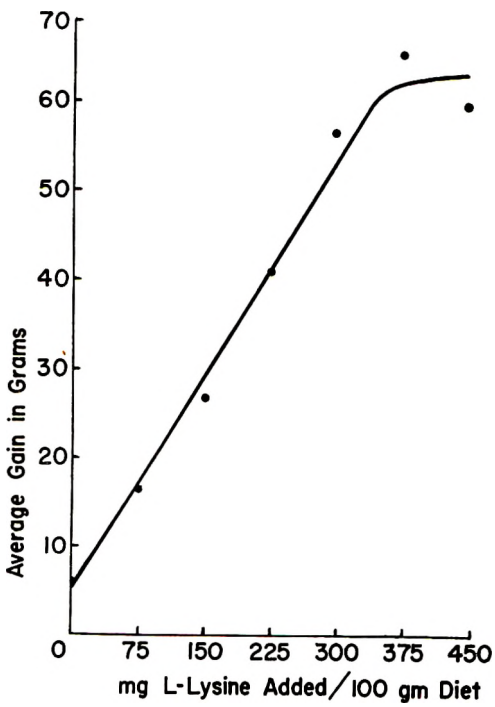

Fig. 1 Average weight gains of weanling male rats fed graded levels of L-lysine (14-day test period).

[6] Holtzman Company, Madison, Wisconsin.

[7] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 2

Quantitative utilization of lysine from meat

Meat sample	Heat treatment	Processing temperature	Lysine[1]	Lysine utilized[2]
		°F	%	%
Beef round				
Raw			9.2	84
Inner portion[3]	Electric oven	200	9.2	82
Outer portion	Electric oven	200	9.7	80
Inner portion[3]	Electric oven	300	9.7	74
Outer portion	Electric oven	300	9.3	88
Inner portion[3]	Electric oven	400	9.6	77
Outer portion	Electric oven	400	9.1	86
Inner portion[4]	Electronic oven	—	9.2	79
Outer portion	Electronic oven	—	9.6	82
	Autoclaved, 4 hours	250	9.2	67
	Autoclaved, 16 hours	250	8.9	37
Ground, + 4% NaCl	Canned, $F_o = 2$	240	9.1	72
Ground, + 4% NaCl	Canned, $F_o = 6$	240	8.9	78
Ground, + 4% NaCl	Canned, $F_o = 10$	240	9.2	61
Ham, uncured				
Raw			11.1	88
Inner portion[5]	Electric oven	300	11.6	90
Outer portion	Electric oven	300	11.6	92
	Autoclaved, 16 hours	250	11.1	53
Lamb leg				
Raw			8.9	88
Inner portion[6]	Electric oven	300	9.0	80
Outer portion	Electric oven	300	9.2	76
	Autoclaved, 16 hours	250	9.2	46

[1] Percentage of lysine in the protein (N × 6.25); lysine determined microbiologically.
[2] Percentage of total lysine utilized for weight gains of weanling rats.
[3] Internal temperature, 140°F.
[4] Internal temperature, 136°F.
[5] Internal temperature, 185°F.
[6] Internal temperature, 170°F.

levels of meat samples were compared with those obtained when graded amounts of L-lysine hydrochloride were substituted to levels of 75, 150, 225, 300, 375 and 450 mg of lysine (calculated for free lysine) per 100 gm of diet. A typical standard response curve is shown in figure 1.

The test samples were substituted in the basal rations at levels to supply 150 and 300 mg of added lysine per 100 gm of diet. The amounts of lysine utilized from the test samples were estimated by comparison of the average weight gains of the animals fed the test samples with the average weight gains of those fed graded levels of the pure amino acid (fig. 1). Percentage utilization expresses the ratio of lysine utilized for weight gain to the microbiologically-determined lysine in the sample. Lysine content as determined microbiologically and the percentage lysine utilization are given in table 2. The values for lysine content of the meat samples agree well with those reported previously by Sherman ('41) and Schweigert et al. ('49).

The observations shown in table 2 indicate that standard cooking procedures do not appreciably affect the utilization of the lysine from muscle meats for growth of the weanling rat. The lysine utilization values obtained from the pork, beef and lamb muscle cuts show that approximately 75 to 90% of the lysine was utilized for growth, either before or after the sample had been subjected to standard conditions of cooking. No consistent differences in lysine utilization were noted between the inner and outer portions of the cooked samples or between the methods of cooking used. Of the canned beef, only the samples receiving the most extensive heat treatment ($F_o = 10$) showed a definite de-

crease in lysine availability. Autoclaving at 250°F and 15 pounds pressure for 16 hours significantly reduced lysine availability in all samples. The autoclaving procedure resulted in little or no loss of lysine as determined microbiologically, but produced a marked decrease in the utilization of the lysine by the animal for growth (table 2). This may be associated with heat-induced chemical changes in the nonpeptidyl amino groups of lysine and arginine in the protein so as to affect the rate of tryptic hydrolysis and therefore the rate at which various amino acids may be made available to the animal for protein synthesis (Gupta et al., '58).

Wheeler and Morgan ('58) studied the rate of appearance of the essential amino acids into the portal blood of weanling rats when fed fresh as compared with autoclaved pork. They found that the time interval for the amino acid to reach maximum concentration in portal blood was approximately the same for each amino acid when fasted animals were fed fresh pork. After they were fed autoclaved pork, however, some amino acids reached their peak concentration in the portal blood several hours later than others. The observed differences in the rate of appearance of certain amino acids in the portal blood of the animals fed the fresh and autoclaved pork may have created the dietary effect of an amino acid imbalance. The possibility exists that this may be the mechanism for the decreased utilization of lysine observed with autoclaved meats.

These studies add to previous information in showing that lysine availability from meats heated under moist conditions is not significantly altered unless severe heat treatment, such as autoclaving, is used.

SUMMARY

The effects of a variety of heat processing methods upon the lysine content of meat protein and its subsequent availability for growth of the weanling rat were investigated. The amount of lysine as determined microbiologically was not affected markedly by any of the heat treatments used. The percentage of available lysine in pork, beef and lamb muscle ranged from 74 to 92% in either raw samples or after standard cooking procedures, indicating standard cooking procedures do not appreciably affect the utilization of lysine from muscle protein. Severe heat treatment (autoclaving for 16 hours) substantially reduced the amount of available lysine in these products.

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Sex Differences in Effect of Restriction of Time of Access to Food on the Plasma Lipid Components in Rats[1]

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In the course of investigating the effects of composition of dietary fats on cholesterol metabolism in young rats, we observed that when certain fats were fed with cholesterol, one to three quarters of the females had serum cholesterol levels which were two to three times as high as those of littermate males or other females fed the same diet. The extent of the sex differences in the level of serum cholesterol seemed to have some relation to the kind of dietary fat, but not to be associated with the presence of any particular known component. Female rats fed safflower, olive, cottonseed, showed the high serum cholesterol values, as did a few of the rats fed coconut oil; those fed corn oil, peanut oil, and butter did not (Okey et al., '59). Moreover, within a group showing the sex difference, part of the females always showed serum cholesterol values distributed around the same median as the males, and part around a higher one; i.e., they fell into two distinct groups.

We had previously found that dosage with estradiol benzoate produced large increases in serum cholesterol values in cholesterol-fed male rats, both intact and castrate. This also decreased food intake to a marked extent (Okey and Lyman, '56). Female rats had been reported to eat little during estrus. We had also observed that restriction of time of access to food might exaggerate the sex differences in the level of serum cholesterol using a given diet, even when total food intakes of the control and of the time-restricted groups were not very different (Okey et al., '60).

In an attempt to find the reason for the above, we investigated the validity of three possible hypotheses: (1) that limitation of time of access to food, i.e., pattern of food consumption might be a primary influencing factor; (2) that a hormone produced in different amounts at different stages of the estrus cycle might be altering the level of serum cholesterol; and (3) that the high serum cholesterol levels in the female rats might be associated with a consistent variation in fatty acid constituents of the cholesterol esters, or of other plasma lipids. The latter was considered of particular interest since the increase of serum cholesterol has been found primarily in the esterified rather than in the free cholesterol fraction.

EXPERIMENTAL

A method for separation of plasma lipids in micro quantities was developed. Plasma lipid extracts from individual rats were transferred into petroleum ether solution, placed on specially prepared silicic acid columns and successively eluted into their component cholesterol esters, triglycerides, mono- and diglycerides and free fatty acids, and phospholipids (Lis et al., '60). Subsequent determinations of the percentages of individual fatty acid in the mixtures which constituted each fraction were found practicable with the available equipment for gas-liquid chromatography.

Groups of weanling rats of both sexes (Long-Evans strain) were caged singly,

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and fed 4 adequate synthetic diets[3] which differed in respect to (a) saturation of the fat and (b) cholesterol content. One subgroup was continued on the ad libitum regimen; the other was allowed access to its diet for only two hours daily (from 8:00 to 9:00 A.M. and 4:00 to 5:00 P.M.). Food intake and weight records were kept for all rats. Size of the subgroups varied from 5 males and 5 or 6 females for the ad libitum groups fed no cholesterol (i.e., on dietary regimens for which we had many data from pervious experiments) to 12 males and 12 females for the rats with restricted access to the cholesterol diets. Cottonseed and coconut oils were chosen as examples, respectively, of preponderantly unsaturated and saturated fats for which sex differences in serum cholesterol levels had previously been observed. The preliminary three-week period of ad libitum feeding had previously been found necessary if the rats were to adjust to a two-hour feeding schedule without difficulty. There were no casualties and no evidence of illness in the present experiment. No evidence of coprophagy was noted, although the use of coarse wire cages and safety food cups were the only precautions taken to prevent it.

Vaginal smears were made on all female rats 24 hours before sacrifice to establish the stage of the estrus cycle. This made it possible to predict within three to 4 hours the terminal stage of the animal. A second smear was made immediately before autopsy in order to confirm the stage at the time of sacrifice. On the basis of the superficial appearance of the genitalia and the types of cells present in the smear, each female was classified into one of 5 stages of the estrus cycle.

Food cups were removed from all animals 15 to 18 hours before autopsy. Rats were anesthetized with pentobarbital sodium, and blood was collected by heart puncture, with heparin as an anticoagulant. The plasma was separated immediately by centrifugation, and 3.0 ml were extracted first with 30 volumes of 95% ethanol at 60°C for one hour, a second time with ethanol: ethyl ether (3:1) for an hour at room temperature and finally with ethyl ether in a Soxhlet apparatus for 14 to 18 hours. The combined extracts were evaporated to dryness *in vacuo* and with a stream of nitrogen and the lipid was redissolved in dry petroleum ether and stored at 0°F until used for final analyses.

The lipid analyses were carried out as follows: each extract was transferred quantitatively to a 15-ml glass-stoppered centrifuge tube and made up to 10 ml at room temperature. A 2-ml aliquot was used for determination of total cholesterol and for a control fractionation of total fatty acids by gas-liquid chromatography—the latter to serve for comparison with the values obtained from the fractions later separated on the silicic acid columns. The analysis of the 2-ml aliquot included saponification with alcoholic KOH, dilution with water, and extraction of cholesterol from the alkaline aqueous phase with petroleum ether. Cholesterol was then determined by the method of Sperry and Webb ('50). The residual soap solution was acidified, the fatty acids extracted, transferred to absolute methanol and methylated with 2% H_2SO_4 as a catalyst. The methyl esters of the fatty acids were subjected to gas-liquid chromatography. Areas of deviation on the resulting graphs were measured with a compensating planimeter. Area factors for individual fatty acids, as well as location of peaks, were checked at least once daily by comparison with graphs made with known mixtures of the most nearly pure fatty acids available commercially.

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[3] *Basal diet:* (in per cent) vitamin-free casein, 5; egg albumin, 10; fat, 10; salts (USP 14), 4; sucrose, 68.6; vitamin A mix, 1.0; vitamin B mix, 1.0; choline mix, 0.4; 1% of cholesterol (when fed) was substituted for sucrose. Fat in the diets coded CSO was cottonseed oil, linoleate content about 54%, oleate 25%; in those coded CN was coconut oil, linoleate content about 3%, oleate 6%. Addition of 1% of cholesterol was coded +C.

Vitamin A mix contained (in grams) per 100 gm: vitamin A distillate (500,000 I. U./gm), 0.35; vitamin D (viosterol), 0.03 (400,000 I.U./gm); α-tocopherol, 0.67; menadione, 0.05; cottonseed oil, 98.9.

Vitamin B mix contained per 100 gm (in milligrams): thiamine·HCl, 40; riboflavin, 40; niacin, 100; Ca pantothenate, 100; *p*-aminobenzoic, 100; folacin, 20; pyridoxine·HCl, 20; biotin, 20; sucrose, 93.56; and (in grams) inositol, 5; ascorbic acid, 1.

Choline mix. Choline bitartrate per 100 gm, 36 gm; sucrose, 64 gm.

Fractionation of a long series of such standards, as well as repeated fractionations of aliquots of large samples of liver lipid extracts, had been carried out previous to this investigation. All of these analyses indicated the reliability of values for individual fatty acids calculated as percentages of total fatty acids in the sample. With our equipment (Wilkins Aerograph) errors in loading the very small samples, differences in performance of columns, lack of facilities for accurate weighing of microgram samples, all contributed to make expression of values for fatty acids as milligrams per 100 milliliters of plasma undesirable.

The remaining 8.0 ml of the petroleum ether extract were evaporated to less than 0.2 ml and the lipid fractions separated by silicic acid chromatography (Lis et al., '60). The individual fractions were placed on a steam bath and evaporated to dryness. At no time were the samples left exposed to air, the last traces of solvent always being removed by a stream of nitrogen, and the lipid dissolved immediately, either in the medium used for saponification or that used for methylation.

Fraction 1, the cholesterol esters, was saponified as described above and the ester cholesterol was extracted from the alkaline aqueous phase. Cholesterol was determined by the Liebermann-Burchard color reaction without digitonide precipitation. The aqueous phase was acidified, and the fatty acids were extracted, methylated, and analyzed by gas-liquid chromatography as described previously.

Fraction 2, the triglycerides, was methylated directly, and the fatty acid esters were extracted and determined by gas-liquid chromatography.

Fraction 3, free cholesterol, free fatty acids, mono- and diglyceride, was treated exactly as fraction 1.

Fraction 4, the phospholipids, was methylated, and the fatty acid esters were extracted and determined by gas-liquid chromatography. The aqueous phase was then digested and the phosphorus was determined (Sumner, '44). It is recognized that transfer of an alcohol-ether extract of plasma to petroleum ether results in considerable diminution of the phosphorus content. Data are therefore presented as petroleum ether-soluble phospholipids.

RESULTS

Growth and food intake. The growth and food intake of all animals followed the pattern shown in figure 1. The body

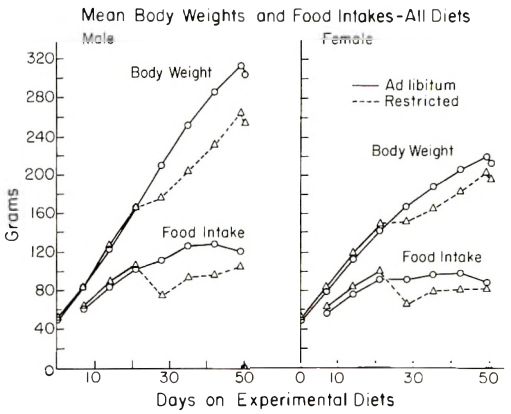

Fig. 1 Food intake and growth of the rats fed ad libitum and those with restricted access to food.

weights reflected the food intake. In the males fed ad libitum, intake leveled off during the 5th week, whereas the intake of the females increased rapidly until the third week and not greatly thereafter. Male and female rats in the time-restricted groups showed a large drop in food intake in the 4th week, when limitation of access to food was begun. Both increased their intake during the 5th week. At the time of sacrifice females were eating approximately as much as their littermates fed ad libitum, males somewhat less than ad libitum-fed males.

Plasma cholesterol. Means and standard errors for plasma cholesterol values are shown in figure 2. "Total" cholesterol values represent data for the total plasma extracts. "Esterified" and "free" cholesterol values are the data for fractions 1 and 3, respectively, from the silicic acid microcolumns.

Total cholesterol values for the rats fed no cholesterol did not differ significantly either with sex or method of feeding. When cholesterol was added to the diet, the males fed ad libitum showed total plasma cholesterol values which did not differ significantly from those of the animals fed no cholesterol (fig. 2). Female rats fell

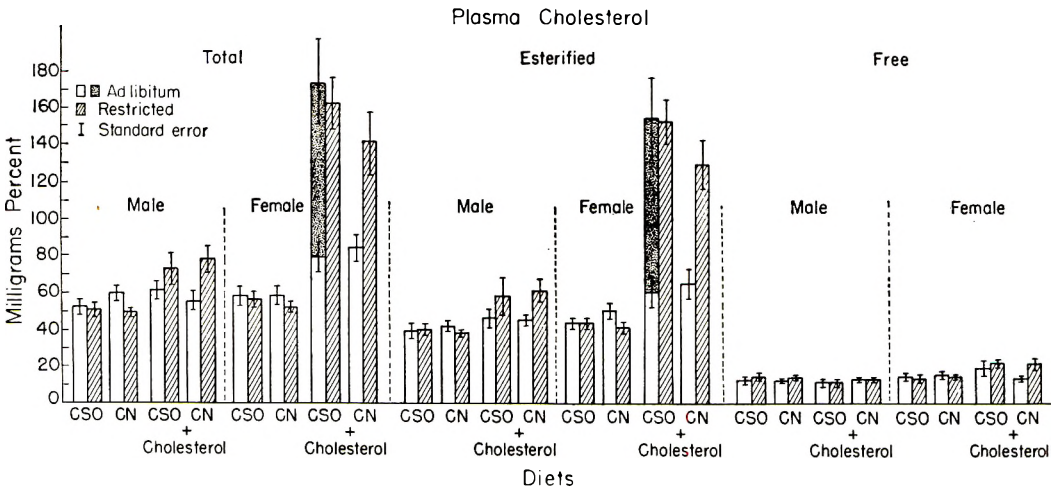

Fig. 2 Total, esterified and free plasma cholesterol values for the different diet groups. Diet groups are indicated as follows: CSO, cottonseed oil only; CN, coconut oil only; CSO + cholesterol, cottonseed oil plus cholesterol; CN + cholesterol, coconut oil plus cholesterol; plain columns represent rats fed ad libitum; hatched columns, those with limited access to food.

into two distinct groups, one with mean plasma cholesterols in the same or a slightly higher range than that for the male rats fed the same diet. When using the cottonseed oil diet, half of the values for females ranged from 116 to 241 mg per 100 ml, with a mean of 174 mg per 100 ml and a standard error of 22. The other half ranged from 52 to 99 mg per 100 ml, mean 76 mg per 100 ml and a standard error of 8. The mean for males was 56 mg per 100 ml and standard error, 8. Two of the 11 samples for females fed coconut oil-cholesterol ad libitum were lost in analyses; one value was 118 mg per 100 ml and the others averaged 83 mg per 100 ml with a standard error of 7. The mean for males was 55 mg per 100 ml, standard error, 7. The coconut oil-fed females differed from one previous group (Okey et al., '59) and from three groups fed the same diet in a later experiment in that, in the other experiments approximately one fourth of the ad libitum cholesterol-fed females showed serum or plasma values over 100 mg per 100 ml, i.e., resembled the cottonseed oil-fed females in the present study.

Values for the cholesterol-fed females with restricted access to food were nevertheless significantly different from all the observed females fed the same diets ad libitum. Total ranges with the cottonseed oil diet were 94 to 227 mg per 100 ml with the middle half of the values between 142 and 173 mg per 100 ml, mean 163 mg per 100 ml, standard error 13; with the coconut oil diet, total range 109 to 286 mg per 100 ml, range of the middle half of the values between 125 and 141 mg per 100 ml, mean 151 mg per 100 ml, standard error 16. "Meal feeding" appeared therefore to have a consistent effect on the distribution curves for plasma cholesterol of the females fed the cholesterol-rich diets.

Only one group of males, those fed coconut oil and cholesterol on a restricted regimen, had significantly higher plasma cholesterol levels than the ad libitum animals on the same diet ($P < 0.05$) or than the rats fed no cholesterol ($P < 0.01$). The male rats fed the restricted cottonseed-plus-cholesterol diet showed cholesterol values which were not significantly higher than those of the males fed ad libitum but were higher than those for the males fed cottonseed oil without cholesterol ($P < 0.05$).

Relatively small variations in *free plasma cholesterol* (fig. 2) were observed in all the rats regardless of diet, feeding regimen or sex. Values ranged from 10 to 16 mg per 100 ml except in a few of the females with very high total cholesterols for which figures reached 20 mg per 100 ml.

Cholesterol ester as eluted from the silicic acid columns with 1% ethyl ether in

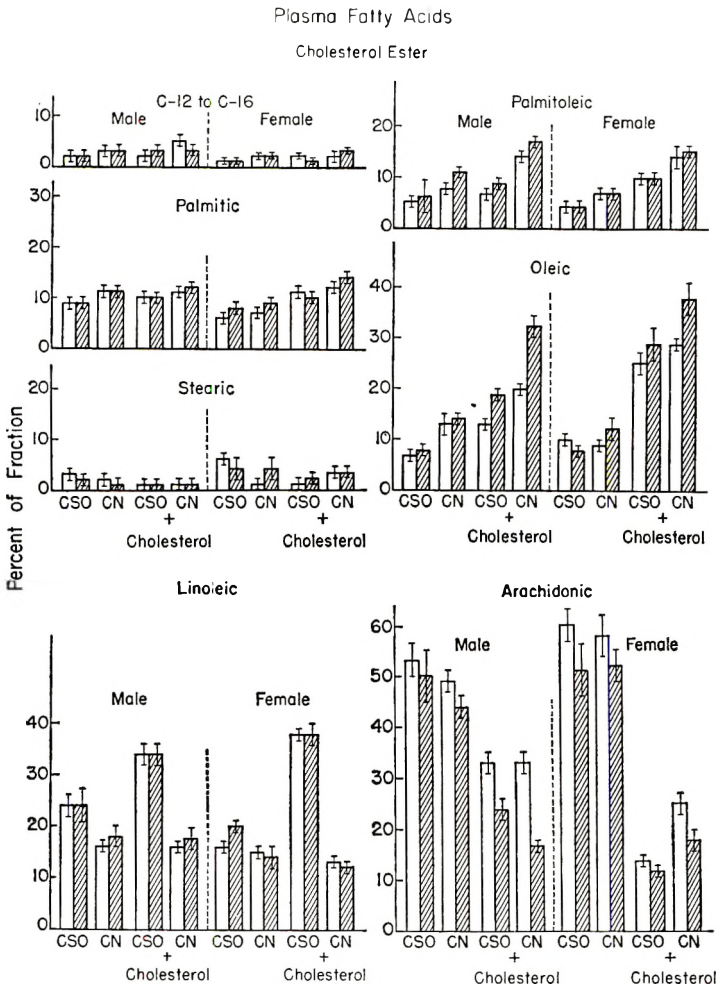

Fig. 3 Plasma cholesterol ester fatty acids expressed as percentages of the totals in the ester fractions from the silicic acid column separation. Data are arranged as in the previous figures.

petroleum ether constituted a cleanly separated fraction of consistent composition. Cholesterol was separated after saponification and the residual fatty acids methylated and chromatographed. Data for the cholesterol ester fatty acids are given in figure 3.

Arachidonic acid constituted a large proportion (50 to 60%) of the fatty acid moiety in the animals of both sexes, provided no cholesterol was fed. With cholesterol feeding, the proportion of arachidonic acid decreased. This was true to a greater extent in the rats with restricted access to food than in the rats fed ad libitum. The change was most marked in females showing high plasma cholesterol values. When the diet furnished linoleic acid (e.g., from cottonseed oil) this acid took the place of the arachidonic acid. Otherwise, the deficit was made up by palmitoleic and oleic acids. The ratio of percentage of unsaturated to saturated acid remained approximately 85 to 15. The rise in cholesterol ester oleic acid was especially great in the females with restricted access to the coconut oil-cholesterol diet, i.e., with a limited intake of linoleic acid and high total cholesterol ester values. Cottonseed oil furnishes considerable amounts of oleic acid as well as of linoleic acid, whereas coconut oil contains little of either acid (that

used in this experiment, about 3% of linoleate and 6% of oleate).

The triglyceride fraction (fig. 4) contained little or no arachidonic acid. The proportion of linoleic acid varied with that in the diet. Oleic and palmitoleic acids were apparently substituted for linoleic acid when the dietary supply of the latter was limited. The proportion of saturated and unsaturated acids in the triglyceride remained more or less constant at 35 to 40% of the saturated and 60 to 65% of the unsaturated.

Data of the *free fatty acid and mono- and diglyceride fraction* indicate the same trend (fig. 5). The size of this fraction in plasma is so small that possible errors in chromatography must be taken into con-

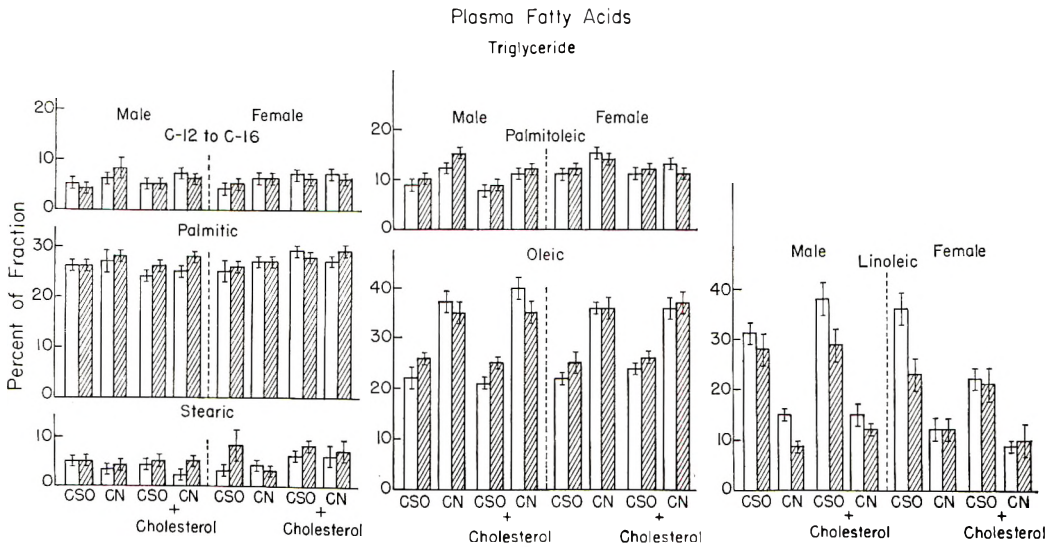

Fig. 4 Plasma triglyceride fatty acids expressed as percentages of the total from the triglyceride fraction from the silicic acid column. Data are arranged as in the previous figures.

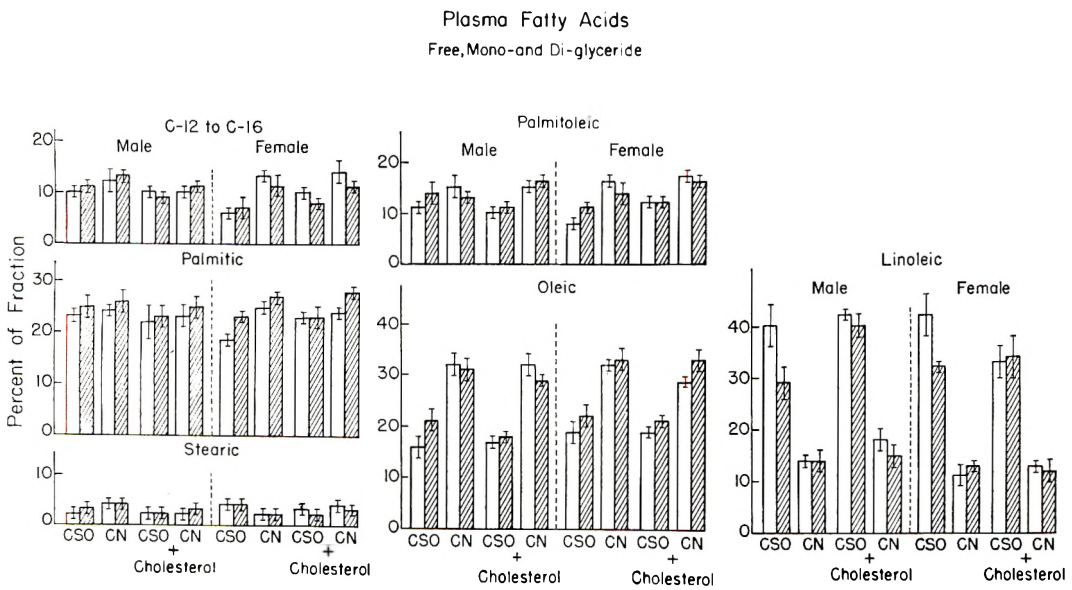

Fig. 5 Free fatty acids, mono- and diglycerides. Data are arranged as in the previous figures.

sideration. The *phospholipid fraction* (fig. 6) contained a larger proportion of palmitic and stearic acids than the cholesterol ester fraction did. There was no very spectacular decrease in percentage of phospholipid arachidonic acid in the cholesterol-fed female rats. In the males, particularly those with restricted intake of the coconut oil diet, percentages of arachidonic acid were low, as were the percentages of linoleic acid. Although the changes in monoenoic acid were in the opposite direction, they did not compensate entirely for the lowered linoleic and arachidonic acids. Percentage ratios of saturated to unsaturated acids were approximately 45 to 55. As noted above there is some question whether the petroleum ether-soluble material placed on the silicic columns represents all of the plasma phospholipid.

As noted in the section on methods, gas-liquid chromatography of the unfractionated total lipids of plasma was done as a check on the above analyses. Values for fatty acids were, within the limits of accuracy of the procedure, about what might be expected for the summation of the fractions. Figures are therefore not presented.

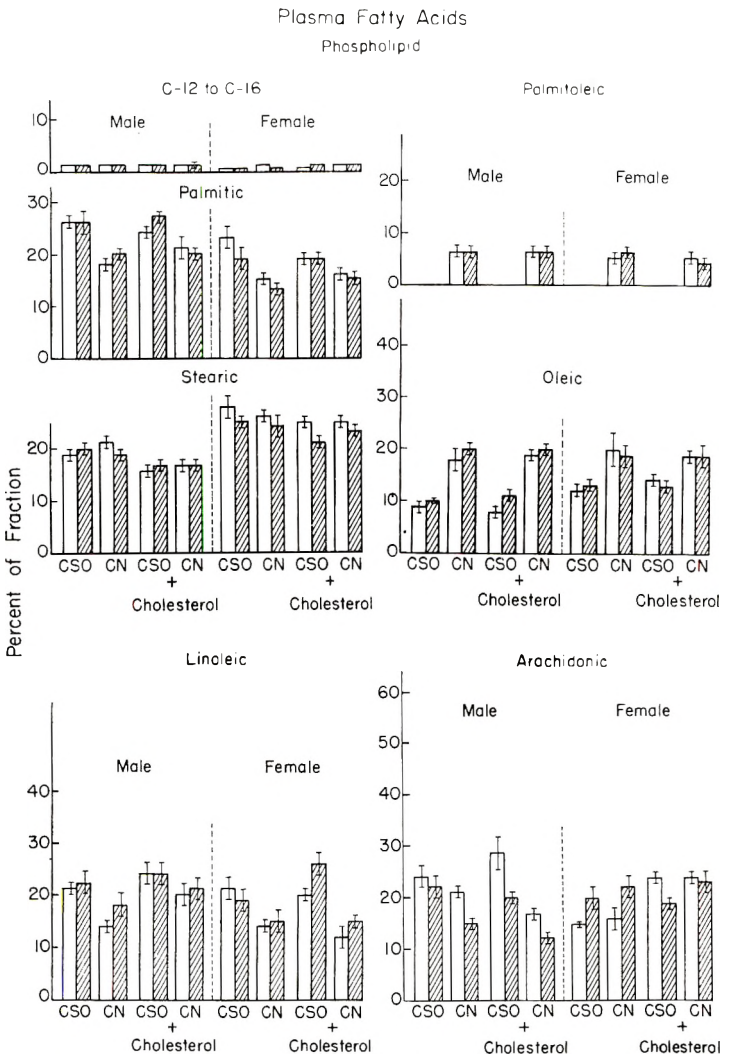

Fig. 6 Plasma phospholipid fatty acids expressed as percentages of the total in the phospholipid fraction. Data are arranged as in the previous figures.

DISCUSSION

Effect of time restriction of food intake. Rats fed ad libitum consume their food gradually throughout the day. With access to food restricted to two hours daily, they tend to adapt to this change by eating larger amounts of food at one time until they approach the total food intake and weight gains of their littermates of the same sex fed ad libitum. In our experiment the females had almost accomplished this by the time of autopsy; the restricted males were still eating a little less.

Cohn and Joseph ('60) reported a study of rats force-fed twice daily. One group was matched to food intake and another to weight gain of ad libitum-fed controls. Evidence was presented for a change in path of carbohydrate metabolism with increased accumulation of fat in the "meal fed" rats. This postulate might account for increased mobilization of fat and cholesterol with "meal feeding."

Sex differences. It is difficult to separate the effects of restriction of access to food and of sex hormones. Apparently the increased size of a cholesterol-containing "meal" led to a higher concentration of cholesterol in the blood of female rats than of males. Male rats, on the other hand, have nearly always shown higher liver cholesterol levels in response to cholesterol feeding than females (Okey et al., '59) but have also shown greater decreases in liver cholesterol on limitation of food intake (Okey et al., '60). Variations of liver cholesterol like those of plasma cholesterol in rats are due primarily to variations in the esterified portion. It is quite possible therefore that sex hormones affect ease of esterification and hence, indirectly, transport and liver storage of dietary cholesterol. However, no consistent relationship between level of liver and serum cholesterol has been evident in our previous studies of ad libitum-fed rats.

Studies with C^{14} labeled cholesterol (Morris et al., '57) have indicated that when cholesterol is fed, the plasma cholesterol is of dietary origin, but that when no cholesterol is fed it is largely endogenous. The small sex variations in plasma cholesterol in the controls of the present series would indicate that there was little or no difference in endogenous cholesterol synthesis due either to sex or to restriction of food intake.

Although females in the present series were autopsied at every stage of the estrus cycle (as indicated by vaginal smears), the number of animals at each stage was too small for differences of the observed magnitude to be significant. The fact that large corpora lutea as well as large follicles are usually present in the rat's ovary at the same time might tend to minimize the dominance of either follicular or luteal hormones. The possibility remains that feeding patterns of some of the ad libitum-fed females may resemble those of the "meal fed" rats.

The fatty acid moiety of the cholesterol ester. Data from this study confirm evidence to the effect that the fatty acids of plasma cholesterol ester are largely unsaturated (Mukherjee et al., '57; and Klein, '57). As noted above, there was little variation in free cholesterol with cholesterol feeding. It was evident, however, that in some way the capacity of the cholesterol-fed animal to supply arachidonic acid for the esterification of cholesterol might be limited. When dietary linoleate was available from cottonseed oil, it seemed to be the preferred substitute. With coconut oil, which supplied little oleate as well as little linoleate, the monoenoic acids seemed to function to maintain a balance of about 85% of unsaturated and 15% of saturated acid in the plasma cholesterol ester. If, as Holman ('58) has suggested, arachidonic acid is the metabolically active acid and linoleic acid is required primarily for conversion to arachidonic acid, the lowering of the percentage of plasma cholesterol ester arachidonic acid by cholesterol feeding may be indicative of interruption of a normal metabolic pathway. Further investigation of the factors which affected relationships between circulating arachidonate and that stored in tissue are obviously indicated.

Decreases in percentage of ester arachidonic acid were significant in cholesterol-fed male rats as well as female and were somewhat exaggerated in animals with restricted access to food. Unfortunately limitations of accuracy of determinations by gas-liquid chromatography are greatest

in the area of the long chain unsaturated acids.

The fatty acid moieties of triglycerides, of mono- and diglycerides, and of phospholipids apparently varied with composition of the dietary fat rather than with sex. Limitation of time of access to food had a much smaller effect on the linoleic acid content of triglyceride than on that of cholesterol ester. The fact that arachidonic acid was found only in cholesterol ester and phospholipid is interesting in relation to its postulated function as an essential fatty acid. Sex differences in plasma cholesterol level did not seem to affect maintenance of a characteristic ratio of saturated to unsaturated acids in each lipid fraction, with monoenoic acids taking the place of the polyunsaturated acids when the latter were in short supply.

SUMMARY

An investigation of plasma lipid components in young adult rats of both sexes is reported. It was designed to find why part of the cholesterol-fed females given certain fats as 10% of adequate synthetic diets had a higher range of esterified plasma cholesterol values than males or than the rest of the females fed the same diets. Cottonseed and coconut oils were chosen as examples of unsaturated and saturated fats.

Limitation of time of access to food resulted in shifting nearly all of the plasma cholesterol levels in cholesterol-fed females into the higher range. Increases in plasma cholesterol values in the restricted cholesterol-fed males were comparatively small. No significant correlation of high cholesterol levels with any phase of the estrus cycle was found. Data did not eliminate the possibility of differences in eating habits of ad libitum-fed females.

Cholesterol feeding resulted, in rats of both sexes, in significant lowering of the percentage of arachidonic acid in plasma cholesterol ester. The change was greatest in the female rats with high cholesterol values and restricted access to food. When linoleic acid was available from the diet, it took the place of arachidonic acid in the cholesterol ester. Without it, i.e., when fed the coconut oil diet, the percentage of oleic acid was increased.

There was a tendency toward maintenance of a relatively constant and characteristic ratio of unsaturated to saturated fatty acids in each of the plasma lipid fractions regardless of diet, sex or feeding pattern. Approximately 85% of the cholesterol ester fatty acids were unsaturated, those of triglycerides, 60 to 65%, of phospholipids about 55%. The data suggest that rate of esterification of excess dietary cholesterol may differ in males and females and that time restriction of access to food may delay removal of cholesterol ester from plasma to a greater extent in females than in males.

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Energy Requirements of Men in Extreme Heat

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One of the missions of this laboratory has been to conduct periodic nutrition surveys in various areas of the United States under extreme environmental conditions and on troops performing a wide range of physical activity. Studies have been completed on men living in a temperate environment (Consolazio et al., '59) and on those living outdoors in a sub-Arctic environment (Welch et al., '57), in both of which the men were performing hard physical work. On the basis of these studies it was concluded that the energy requirements in temperate and sub-Arctic environments were essentially the same, providing the men were clothed adequately.

These results are contrary to the work of Johnson and Kark ('47) who published data showing that the energy requirements were inversely proportional to the environmental temperature. As a result of their work, the revised dietary allowances recommended by the National Research Council ('58), and the Food and Agriculture Organization ('57), were adjusted for climatic differences.

Several recent studies have been reported of the effects of high environmental temperature on the caloric intake of men. A preliminary study was conducted on troops living and working in the desert heat for two weeks (Consolazio et al., '60). The daily food consumption for a headquarters company averaged 4061 Cal. and for a military police company 4416 Cal. per day. In this study the body weight changes in both groups were negligible. These unusually high food intakes encouraged a second study which was designed to determine the effect of solar radiation and temperature *per se* on the energy expenditure and caloric requirements of men.[1]

In this study the daily energy requirements, corrected for body weight changes using Keys and Brozek factors ('55) were significantly increased in both the sunlight and high temperature phases when compared with the cool indoor phase.

The present study conducted in the desert heat at Yuma, Arizona, was designed to answer some of the pertinent questions related to the energy requirements of men living and working in the heat. The effects of solar radiation and extreme heat on the energy requirements of men performing a constant daily activity were to be evaluated by (a) daily energy expenditure and energy balance, (b) fluid and nitrogen balances, (c) sweat rates, (d) body temperature changes, (e) daily body weight changes, and (f) body composition changes.

EXPERIMENTAL

The study was divided into three 10-day experimental periods using 8 normal, healthy, young conscientious objectors as test subjects. During period 1 the men were kept outdoors in the direct sunlight from 7 A.M. to 5 P.M. daily. In period 2 the men were also outdoors, but were kept in the shade under a large tarpaulin, and during period 3 the men were moved indoors into an air-conditioned room. In all periods, the men lived in air-conditioned barracks at 26°C from 5 P.M. to 7 A.M. daily. Three days prior to the beginning of the test, the men began to acclimate by training outdoors in the desert heat. The daily activity level was

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[1] Shapiro, R., and C. F. Consolazio 1959 Energy requirements of men exposed to solar radiation and heat. Federation Proc., *18:* 583 (abstract).

kept constant throughout the entire experiment. Food, which was supplied ad libitum during the three meals, was weighed by individual item for each man, and only measured soft drinks and an occasional measured beer were allowed after the evening meal.

For convenience a normal army garrison ration was fed during the entire study. The caloric content of the food consumed was calculated using the chemical analysis data of the food composites (Consolazio et al., '56) which has been used in previous studies by this laboratory. Chemical analyses performed on the food composites included those for nitrogen, fat, moisture, ash and total energy, using bomb calorimetry (Consolazio et al., '59, '60). In addition, chemical analyses were also performed on many of the individual food items served during the study. In this manner the daily food consumption could also be calculated on an individual basis. Metabolizable energy was then computed on the food composites using the Atwater general factors of 4, 9 and 4 for protein, fat and carbohydrate, respectively. Gross and metabolizable energy were also computed using the food consumed and excreta data obtained from bomb calorimetry.

The energy cost of the various daily activities was measured both morning and afternoon by means of indirect calorimetry using the Müller-Franz metabolimeters (Insull, '54). Activities measured included walking on the treadmill for one hour on the level at 4.0 mph, riding the bicycle for 90 minutes, with no added weights, and the various resting activities. Basal metabolic rates were always determined in an air-conditioned room. Time-motion studies recorded by diary for the evening activities and the measured daytime energy expenditures, were used for determining the total daily expenditure.

Since body weight changes were an integral part of the study, they were taken in duplicate to the nearest 0.01 kg. The men were weighed 5 times daily, (1) in the nude after voiding, immediately upon arising in the morning, (2) after breakfast, (3, 4) before and after lunch, and (5) at the end of the working day, before dinner.

Blood volume (Campbell et al., '58), hemoglobin, hematocrit and plasma proteins (Phillips et al., '50) were measured at the beginning and end of each 10-day test period. Other pertinent data collected included daily fluid (Adolph et al., '47), nitrogen and energy balances.

Pulse rates, blood pressures and body temperatures (rectal) were measured immediately upon arising in the morning, before the noon meal, and at the end of the working day. Meteorological data recorded hourly included indoor and outdoor temperatures, soil and black ball temperatures, relative humidity, wind velocity, solar radiation and barometric pressure.

Urine was collected on a 24-hour basis from each of the 8 men. At the end of each day the volumes were recorded, the specific gravities measured and 10% aliquots saved to be pooled at the end of each period for each man. Fecal samples were collected by periods, using whole corn as a marker. The sample collection began at the first appearance of the corn in the stool. At the end of each period, the samples of each man were combined and the total weight recorded. Aliquots of urine and feces were saved for the subsequent chemical analysis of moisture, ash, fat, nitrogen and total energy.

RESULTS

The average daylight temperatures were 40.5°C for period 1 in the hot sun, 40.3°C during period 2 in the hot shade, and 26°C for period 3 in the cool shade. The relative humidity averaged 30.3, 48.0 and 58.5% for the same periods, respectively. The evening indoor temperatures were fairly constant during each period, averaging 24.1, 24.3 and 26.1°C, respectively.

Body temperatures in period 3 were consistently lower than in the other two periods. Even though the evening body temperatures increased only 0.7 and 0.5°C for periods 1 and 2, they were significantly different from period 3 ($P < 0.001$). The daily patterns for pulse rates and blood pressures were not significantly different.

The daily food consumption data comparing the calculated and the analyzed values by bomb calorimetry are presented in table 1. The calculated values are higher than those derived by actual anal-

ysis. Using values by either method, the food intakes for period 3 were significantly lower than those for either period 1 or 2 ($P < 0.005$). Since the values by analysis are the most reliable, they will be used hereafter in the text.

Outside the mess, food consumption, which consisted only of soft drinks and an occasional beer, averaged 211, 137 and 110 Cal. per man per day for three periods. Because the 10th day in each period was set aside for basal metabolic rates and blood drawing, only two meals were served on these days.

The daily morning body weight changes were small for each period, showing an average gain of 62, 36 and 17 gm per man for the periods 1, 2 and 3 (table 1). The blood volumes showed an average increase of 224 ml (6664 to 6888) in period 1, a decrease of 212 ml (6688 to 6676) in period 2, and a decrease of 20 ml (6676 to 6656) in period 3. Hemoglobin, and serum proteins were not significantly different for all periods, but the hematocrit values showed a significant decrease of 2.5% (48.7 to 46.2) at the end of period 1 and a significant increase of 1.8% (46.2 to 48.0) in period 2.

TABLE 1

Energy metabolized,[1] not corrected for body weight changes

Period	1—Hot sun		2—Hot shade		3—Cool shade	
Subject	Intake	Δ Weight[2]	Intake	Δ Weight[2]	Intake	Δ Weight[2]
	Cal./day	*kg*	*Cal./day*	*kg*	*Cal./day*	*kg*
1	2619	−0.34	2804	−0.29	2386	−0.93
2	3368	+0.44	3551	+0.60	2948	+0.12
3	3539	+0.43	3493	+0.65	3033	+0.36
4	3157	+0.52	3539	+0.97	3295	+0.20
5	3016	+0.59	2798	−0.36	2586	−0.14
6	3782	+0.63	3341	−0.44	3302	+0.63
7	4236	+1.89	4258	+0.74	3789	+0.63
8	4682	+0.80	4326	+1.03	3595	+0.49
Mean, analyzed values, M.E.	3560[3]	+0.62	3516[4]	+0.36	3156	+0.17
Calculated values	3925[3]		3817[4]		3520	

[1] Gross energy of diet less energy in excreta, using bomb calorimetry.
[2] Weight changes are for 10-day periods.
[3] Difference between periods 1 and 3 is highly significant ($P < 0.005$).
[4] Difference between periods 2 and 3 is highly significant ($P < 0.005$).

TABLE 2

Energy cost of various activities

Activity	Periods			Significant differences	
	1—Hot sun	2—Hot shade	3—Cool shade	1 vs. 3	2 vs. 3
	Cal./hour	*Cal./hour*	*Cal./hour*		
Exercycle[2]	223	202	189	$P < 0.005$	$P < 0.025$
Exercycle[2]	240	222	201	$P < 0.005$	$P < 0.050$
Treadmill	341	353	328	N.S.[1]	$P < 0.025$
Resting	102	97	94	$P < 0.050$	$P < 0.025$
Resting, after Exercycle	106	111	97	$P < 0.025$	$P < 0.005$
Resting, after treadmill	101	95	93	$P < 0.050$	N.S.
Basal M. R. (cool shade)	74	75	76	N.S.	N.S.

[1] N.S. indicates not significant.
[2] Exercycle Corporation, New York.

The fluid intakes averaged 7927, 6459 and 3648 gm per man per day for periods 1, 2 and 3, respectively. The fluid output (urine, feces, sweat) averaged 7807, 6338 and 3703 gm per day for the same periods. The net fluid balance for the three periods averaged + 120, + 121 and − 55 gm per man per day. The sweat rates, including insensible water loss, averaged 6382, 4900 and 2050 gm per day. All differences between periods are highly significant.

The nitrogen balance for the three test phases averaged − 0.43, − 0.67 and +1.38 gm of nitrogen per day. A correction factor for nitrogen in sweat was used in calculating the nitrogen balance (Mitchell et al., '49).

Energy expenditure was measured every other day for the various activities, including the treadmill, bicycle and the resting activities. For most activities, the metabolic rates were significantly different when comparing period 1 and 3, or period 2 and 3 (table 2).

The daily energy expenditure of each man for each period was computed on the basis of actual energy cost of the daytime activities and by the calculation of time-motion studies during the evening activities. In calculating the daily energy expenditure, a factor was included for the energy expended in warming cold drinking water. These values increased the daily expenditure by 149, 113 and 48 Cal. per day for each period. The daily energy expenditure averaged 3517, 3439 and 3196 Cal. per day. The energy balance amounted to +43, +77 and −40 Cal. per day for the three test periods, respectively (table 3).

The body weight changes were corrected for body composition changes using fluid balance data and showed an average body weight loss of 58 gm per day for period 1 and 85 gm for period 2. In period 3 there was an average weight gain of 38 gm per day. These body weight changes were further corrected for the nitrogen balance,

TABLE 3

Energy balance

Energy	Subject no.								Mean
	1	2	3	4	5	6	7	8	
				Period 1—hot sun					
Gross intake	2850	3627	3801	3447	3257	4095	4579	5010	3843
Excreted	231	259	262	290	241	313	343	328	283
M.E.	2619	3368	3539	3157	3016	3782	4236	4682	3560
Expended	3153	3122	3134	3440	2814	3982	3504	3792	3368
Cold water[1]	133	170	127	154	133	147	175	171	149
Total expended	3286	3292	3261	3594	2947	4129	3679	3963	3517
Balance	−667	+76	+278	−437	+69	−347	+557	+719	+43
				Period 2—hot shade					
Gross intake	3012	3824	3739	3800	3036	3575	4560	4672	3778
Excreted	208	273	246	261	238	234	302	346	262
M.E.	2804	3551	3493	3539	2798	3341	4258	4326	3516
Expended	2885	3075	3140	3441	2811	3875	3490	3895	3326
Cold water[1]	97	116	88	117	95	108	152	133	113
Total expended	2982	3191	3228	3558	2906	3983	3642	4028	3439
Balance	−178	+360	+265	−19	−108	−642	+616	−133	+77
				Period 3—cool shade					
Gross intake	2586	3165	3277	3543	2809	3494	4106	4174	3394
Excreted	200	217	234	248	223	192	317	279	238
M.E.	2386	2948	3033	3295	2586	3302	3789	3895	3156
Expended	2756	2914	2973	3289	2650	3672	3390	3537	3148
Cold water[1]	36	52	31	48	44	31	92	46	48
Total expended	2792	2966	3004	3337	2694	3704	3482	3583	3196
Balance	−406	−18	+29	−42	−108	−402	+307	+312	−40

[1] Energy utilized for warming cold drinking water.

TABLE 4

Energy requirements (averages of 8 men per day)

	Period		
	1—Hot sun	2—Hot shade	3—Cool shade
Food consumption, M.E. by bomb calorimetry, Cal.	3560	3516	3156
Body weight change, gm	+62	+36	+17
Water balance, gm	+120	+121	−55
Weight change not due to water retention or loss, gm	−58	−85	+38
Protein balance (N × 6.25), gm	−2.7	−4.2	+8.6
Weight change not due to water or protein (due to fat), gm	−55	−81	+47
Caloric equivalent of this weight change, 9 Cal./gm	−498	−729	+423
Energy requirement[1]	4058	4243	2733

[1] Energy requirement if weight change is assumed to be glycogen: (period 1) 3780, (period 2) 3840, (period 3) 3334.

so one may assume that the remaining weight change was primarily due to fat. This we do not really know but workers in the field assume that if water and nitrogen are accounted for, then only fat is laid on. This may not be true. The energy equivalent of these body weight changes were + 498, + 729 and − 423 Cal. per day for the three periods, making the energy requirements 4058, 4243 and 2733 Cal. per man per day for periods 1, 2 and 3, respectively (table 4). These requirements were equivalent to 55.5, 56.4 and 36.6 Cal. per kg of body weight, respectively.

DISCUSSION

As mentioned previously the NRC Committee on Dietary Allowances ('58) and the FAO Committee on Caloric Requirements ('57) have recommended a decrease in caloric intake with an increase in environmental temperature. The NRC Committee suggests a decrease in caloric requirements of 5% for every 10°C above the base temperature of 20°C. The FAO Committee uses a 5% decrease in requirements for every 10°C above the base temperature of 10°C. Using the NRC and FAO formulae, corrected for temperature differences, the energy requirements of a man living at 40.3°C, weighing 74.5 kg, would be 3037 and 2865 Cal. per day, respectively.

Since the recommended allowances seem to be opposite to the findings observed in the present study, a re-evaluation of the energy requirements in extremely hot environments is necessary. This is especially true because very few of the reported studies are comparable, in that many of the conditions have not been strictly controlled. The use of various body composition factors for the calculation of the energy equivalent of changes in body weight have been the most obvious. In one study (Welch et al., '58) the Keys and Brozek factors are used; in another, body density and body water data have been applied (Welch et al., '57), even though the body weight changes have been small; and in some instances the skin-fold-thickness data has been used as a measure of body fat changes (Welch et al., '56).

Mitchell et al. ('49) feel that the energy requirements are decreased in the heat due primarily to (a) lowered basal energy expenditure, (b) a greater efficiency in certain types of work associated with light clothing or a lessened capacity for work, and (c) reduced work performance owing to lessened motivation.

Welch et al., ('58) and LeBlanc ('58) have shown that there is essentially no increase in food intake of men in any environment, except for the 4 to 5% increase in the cold, due to the "hobbling" effect of the heavy clothing.

Recently, Consolazio et al. ('60) and Shapiro and Consolazio[2] suggested that there is an increased requirement in the heat, due primarily to the increased heat

[2] See footnote 1.

load imposed upon the temperature-regulating mechanism of the body.

In the present study, where work was maintained at a constant rate, the voluntary food intake was increased significantly during both the hot sun and the hot shade periods as compared with the cool shade period. These food intakes were considerably higher than the FAO–NRC minimal requirements of 3069 Cal. per day for men living and working in the heat. When corrections are made for changes in body water and nitrogen balance, these increases in energy requirement are even more significant. The water gains in period 1 and the water losses in period 3 (from water balance data) are also supported by proportionately similar changes in the blood volumes and blood hematocrits. (The blood volumes increased and the hematocrits fell in period 1, with the reverse being true in period 3.)

The extremely high intake (4243 Cal.) in period 2 after correction for body weight and body composition changes, may require an explanation. A considerable amount of the daily increase in water balance (121 gm) during this period was due primarily to the unexplained values of one man who had a positive balance of 431 gm. All the other men had balances below 125 gm. By elimination of this one subject, the weight change equivalent would be 424 Cal., making the corrected food intake 3940 Cal. per day, which would be in line with the values in period 1.

The protein intake was considerably higher than the NRC recommended allowances of 100 gm a day for all three test periods. This is in agreement with Mitchell's study ('49) showing an increased requirement for protein in the heat. Mitchell concluded that the nitrogen losses in the sweat are uncompensated by diminished losses in the urine. We feel that the increased protein consumption is due primarily to the increased caloric intake and not to the increased requirement.

The fat intake was approximately the same for all three phases, ranging between 37 and 40% of the total calories consumed. These values are in the same range as those found in temperate (Consolazio et al., '59) and extremely cold environments (Welch et al., '57). One can then assume that the fat intake is a matter of choice, since it does not seem to be affected by the environmental temperature.

The increase in food intake in the heat could also be explained by the increase in energy expenditure alone, which averaged 3517, 3439 and 3196 Cal. per man per day for periods 1, 2 and 3, respectively.

The significant increases in metabolic rate, which are in agreement with other studies in the literature, could account for the increased food intake. In the present study the metabolic rates during the two exercycle activities were increased by 18.0 and 19.4% when performed in the hot sun, whereas for the resting activities they were increased by 8.5 to 9.3%. Welch et al. ('56) and Shapiro and Consolazio[3] have shown that there is a significant increase in resting metabolism in the heat, of 35 and 21.8%, respectively. The latter investigators have observed an increase of 26.6% in the total metabolic rate of men working on an exercycle at a constant rate in extreme heat as compared with that in a cool environment. Herrington ('40), in his work on small animals, reports an increase in metabolic rate in both the heat and the cold, when compared with that in a temperate environment.

Eichna et al. ('40) and Christensen ('33) have shown that there is an increase in total metabolic rate of 11 to 13% and of 10.8% respectively, for each degree rise in body temperature. Christensen ('33) feels that this is partly owing to the increased activity of the heart, lungs and glands, but is primarily the result of increased cellular metabolism.

The average difference in body temperature between periods was 0.7°C and even though the values seemed small, they were highly significant. Using an average value of 11.6% increase in total metabolic rate for each degree centigrade rise in body temperature, a 0.7°C rise would be equivalent to an 8% increase in metabolic rate. This 8% increase in metabolic rate is due primarily to the increased food intake observed during the hot periods. Other sources of heat gain, solar radiation and heat by convection, add approximately 150

[3] See footnote 1.

Cal. per hour to a clothed man standing in the sun according to Adolph et al. ('47). These investigators have shown that shade is a valuable protection against radiant energy, since a man will expend 100 Cal. per hour less in the shade than in the sun. In our comparisons body temperatures in the hot sun and in the hot shade differed little since the men wore light cotton pajamas for protection against the sun.

The significant differences in food consumption, during the hot sun and hot shade phases, appear to be due to the increased work load imposed on the temperature regulating mechanism of the body with exposure to sunlight and high temperatures. The operative mechanism by which the body loses heat is circulation of the blood to the skin surface. Since the skin temperature is usually 33°C, heat transfer will take place by radiation, conduction and convection, if the surrounding air and ground temperatures are below 33°C. When temperatures are above 33°C, man has only one method of dissipating heat. This is by the evaporation of water or sweat (evaporative cooling), and is the underlying principle which allows man to exist in the desert. By evaporation, water cools the skin and in turn the blood, which brings the heat to the surface. The energy required to evaporate one gram of sweat (or water) from the surface at 30 to 40°C is equivalent to 0.58 Cal. (Adolph et al., '47). This is the latent heat of vaporization of water and when applied to man includes all the water vaporized at such a temperature, including that from the lungs. The source of calories for the vaporization of sweat may be the body or the immediate environment surrounding the body, and will depend upon many factors, including surface area, conductivity, wind velocity and humidity. Adolph et al. ('47) have shown that the sweat rate increases 40 gm per hour for each 1°C rise in air temperature and that direct sunlight is responsible for an elevation in sweat rate roughly equivalent to a rise in air temperature of 5°C, or 200 gm per hour. This holds true in this study since the sweat rates during the sun phase were approximately 1500 gm more than in the hot shade period, or a difference of 167 gm per hour.

The important factor to consider is that the body must maintain heat equilibrium, whether the caloric source for vaporization of water be the body or the environment. The heat lost by the body must equal the environmental heat load plus the metabolic heat load. If one assumes that environmental heat does not evaporate water directly from the skin, then the heat lost from the body is equal to skin water loss multiplied by the latent heat of vaporization. Only if it is further assumed that the metabolic heat load is small in relation to the environmental heat load might one expect the sweat rate to be in proportion to the environmental heat load.

Since corresponding increases in food intake, metabolic rate, fluid intake and sweat loss occurred in periods 1 and 2, it appears that the sweating process plays an important role in determining the energy requirements of men exposed to solar radiation or high temperatures. The relationship of the sweating process to the energy requirements of men may be attributed to several factors. At high temperatures or when exposed to sunlight, an increased work load is imposed upon the temperature regulating mechanism of the body. For example, the transport of heat by the blood may require more work and energy. Likewise the sweat glands themselves perform more work.

In all likelihood, it is a combination of increased action by the blood in heat transport, increased action of the sweat glands, and increased body temperature, which are causing an increased caloric requirement in environments inducing these mechanisms of temperature regulation.

The data in this study suggest that there is a significant increase in the energy requirements of men living and working in extremely high temperatures and in the sun. On the basis of the results in this study, it would appear that the NRC and FAO minimal allowances should be reevaluated, especially in view of their recommended decrease in energy intake for men living in a hot environment.

SUMMARY AND CONCLUSIONS

A study was performed in extreme heat on 8 healthy young adults for three consecutive 10-day periods. In period 1 daytime temperatures in the hot sun averaged 40.5°C, in period 2 in the hot shade, 40.3°C, and in period 3 in the cool shade 26.0°C. The men carried on constant daily activity, and were allowed food and water ad libitum.

1. With the set conditions of this study, the data suggest that there is an increased caloric requirement for men working and living in extreme heat. Significant increases were observed in food consumption and the actual caloric requirements were even greater because of changes in the body composition of the men.

2. The differences in energy cost of the various resting and exercise activities, when comparing the hot-sun or hot-shade to the cool-shade phase were significant.

3. Energy requirements averaged 55.5, 56.4 and 36.6 Cal. per kg of body weight, when corrected for body composition changes.

4. These increased requirements are probably due to the increased heat load imposed on the body by solar radiation and extreme heat. The increased requirements are, in all likelihood, a combination of increased action of the blood in heat transport, increased action of the sweat glands, plus the increased total metabolic rate due to the elevation in body temperature.

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Studies on the Vitamin K Requirement of the Chick

II. EFFECT OF SULFAQUINOXALINE ON THE QUANTITATIVE REQUIREMENTS OF THE CHICK FOR VITAMIN K_1, MENADIONE AND MENADIONE SODIUM BISULFITE[1]

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The anti-vitamin K effect of sulfonamides, including sulfaquinoxaline, has been reviewed by Nelson and Norris ('59). In the literature reviewed, however, and in experimental work reported by the authors, no attempt was made to determine the quantitative effect of sulfaquinoxaline on the vitamin K requirement of the chick. Frost et al. ('56) reported that, on a weight basis, menadione sodium bisulfite, USP, was 6 to 10 times more effective than menadione in the presence of 0.1% of dietary sulfaquinoxaline. This was thought to be due in part to the better absorption of menadione sodium bisulfite. Mushett and Seeler ('47) reported that, in dogs, vitamin K_1 was 100 to 250 times more effective on a weight basis than menadione in preventing the hypoprothrombinemic action of sulfaquinoxaline.

Therefore, experiments were conducted to study further the effect of 0.1% of sulfaquinoxaline on the quantitative vitamin K_1, menadione and menadione sodium bisulfite, USP (MSB), requirements of the chick.

EXPERIMENTAL

Male White Plymouth Rock chicks were used in these experiments, with 15 chicks in each lot. The basal diet and experimental procedures used have been reported earlier (Nelson and Norris, '60).

To ascertain the extent to which sulfaquinoxaline increased the vitamin K_1, menadione and MSB requirements of the chick, it was necessary to determine in each experiment the requirement of the chick for the form of vitamin K under study both in the presence and absence of sulfaquinoxaline. Therefore, two groups of chicks were used, one of which was fed diets with graded levels of vitamin K to establish the reference requirement. The second group was fed graded levels of the same form of the vitamin in diets with 0.1% of sulfaquinoxaline to determine the requirement in the presence of this drug. The requirements were then compared to establish the effect of sulfaquinoxaline. All experiments were subjected to analysis of variance as outlined by Snedecor ('56). Differences between individual means were tested by Duncan's test (Federer, '55) and effects of different treatments were analyzed whenever possible by individual degree of freedom comparisons.

RESULTS AND DISCUSSION

Experiments 1 and 2 were conducted to determine the effect of sulfaquinoxaline on the vitamin K_1 requirement of the chick. In experiment 1, one group of chicks was fed diets containing 100, 200, 400, 600 and 800 μg of vitamin K_1 per kg to establish a reference requirement for this vitamin. The second group was fed diets containing 0.1% of sulfaquinoxaline and 400, 800, 1200 and 1600 μg of vitamin K_1 per kg.

The average prothrombin times of the chicks fed the diets without sulfaquinoxaline and containing the increasing amounts of vitamin K_1 were 43.0, 31.5, 26.1, 24.4

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and 25.7 seconds at three weeks and 39.1, 27.2, 23.8, 23.0 and 23.9 seconds at 4 weeks. The three- and 4-week average prothrombin times of the chicks fed the diets with sulfaquinoxaline and containing the graded levels of vitamin K_1 were 58.6, 41.0, 35.3 and 42.0 seconds and 45.0, 31.4, 29.5 and 26.5 seconds, respectively. The prothrombin times of chicks fed diets with sulfaquinoxaline were prolonged at all levels of vitamin K_1 at three and 4 weeks. The effect of sulfaquinoxaline was greater at three weeks than at 4 weeks.

The quantitative requirement for vitamin K_1 at three weeks was 455 μg per kg of diet. In the presence of sulfaquinoxaline the requirement was 10,480 μg per kg of diet. The latter calculation was made assuming that the base line for the two groups of chicks would have been the same if sufficient vitamin K_1 had been supplied the group of chicks fed the diets containing sulfaquinoxaline. The quantitative requirement at 4 weeks was 385 μg per kg of diet. The requirement, in the presence of sulfaquinoxaline, was 2590 μg per kg of diet assuming that minimum prothrombin times could have been attained with sufficient vitamin K.

It was then necessary to determine whether the above assumptions were true. In experiment 2 one group of chicks was fed diets with 90, 180, 360 and 720 μg of vitamin K_1 per kg to establish the reference requirement. The second group of chicks was fed diets with 0.1% of sulfaquinoxaline containing 250, 500, 1000, 2000, 4000 and 8000 μg of vitamin K_1 per kg. No prothrombin determinations were made at 4 weeks on the chicks fed the diet containing sulfaquinoxaline and 250 μg of vitamin K_1 per kg because an insufficient number survived the two-week determinations.

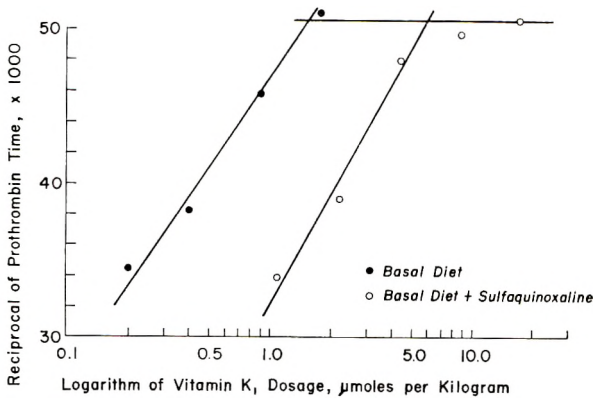
The results are presented in table 1. At two weeks the prothrombin times of chicks fed diets with sulfaquinoxaline were not reduced to normal even with 8000 μg of vitamin K_1 per kg. In contrast, at 4 weeks the prothrombin times of chicks fed the diets containing the two highest levels of vitamin K_1 approached normal. Duncan's test for comparison of individual means showed that the prothrombin times of chicks fed diets containing 720, 4000 and 8000 μg of vitamin K_1 per kg were not significantly different. Therefore, the reciprocals of the prothrombin times of these three groups were used to establish the base line at 4 weeks.

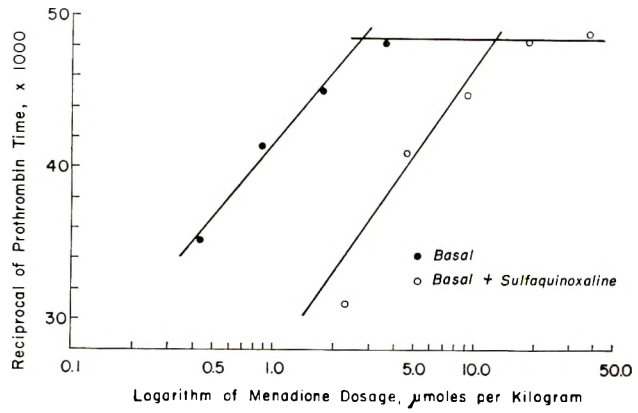
The requirement for vitamin K_1 in experiment 2 was 660 μg per kg of diet at two weeks. Assuming that the base lines of normal chicks and chicks fed diets with sulfaquinoxaline would have been the same at two weeks had sufficient vitamin K_1 been supplied, the requirement was 15,690 μg per kg of diet. At 4 weeks the requirement for vitamin K_1 was 700 μg per kg of diet. The requirement was increased to 2750 μg per kg of diet with the addition of sulfaquinoxaline. The base lines for these two groups of chicks were the same at 4 weeks.

TABLE 1

Effect of sulfaquinoxaline on the vitamin K_1 requirement of the chick (exp. 2)

Vitamin K_1	Av. weight 4 weeks	Feed/gain	Prothrombin time	
			2 weeks	4 weeks
μg/kg diet	*gm*		*seconds*	*seconds*
		Basal diet		
90	519	1.71	31.2	29.5
180	540	1.71	26.9	26.4
360	555	1.69	22.3	21.9
720	573	1.66	20.0	19.4
		Basal diet + sulfaquinoxaline		
250	—	—	63.6	—
500	505	1.78	40.0	29.8
1000	494	1.74	37.3	25.8
2000	514	1.70	27.2	20.8
4000	486	1.78	24.1	20.4
8000	487	1.72	23.7	19.8





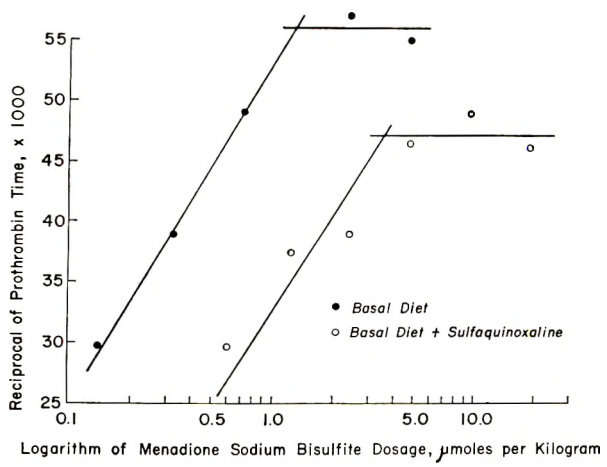


Fig. 1 Effect of sulfaquinoxaline on the vitamin K_1, menadione and menadione sodium bisulfite requirements of the chick at 4 weeks.

A plot of the logarithm of dose against the reciprocal of the 4-week prothrombin times to give the regression lines and the base line is shown in figure 1. The effect of sulfaquinoxaline was to shift the regression line to the right showing an increased need for vitamin K_1. Although the 720 μg level of vitamin K_1 appears to fit along the regression line, it was assumed to represent a normal prothrombin time. Evidence obtained in previous and subsequent experiments shows this to be true. A plot of the two-week data in this experiment and the three- and 4-week data in experiment 1 also gave parallel regression lines.

Experiments 3 and 4 were conducted to determine the degree to which sulfaquinoxaline increased the menadione requirement of the chick. In experiment 3 the chicks were fed diets with and without sulfaquinoxaline containing 76, 152, 305, 610 and 1220 μg of menadione per kg.

The average prothrombin times of the chicks fed diets without sulfaquinoxaline were 28.1, 22.2, 21.4, 19.5 and 19.5 seconds at two weeks and 29.0, 22.8, 21.9, 20.9 and 20.6 seconds at 4 weeks for the graded levels of menadione. The average prothrombin times of the chicks fed diets with sulfaquinoxaline were 130.3, 73.7, 46.5, 35.1 and 36.7 seconds at two weeks from the lowest to the highest level of menadione fed. No prothrombin determinations were made at 4 weeks on the chicks fed the diet containing 76 μg of menadione per kg because of the high rate of mortality following the two-week determinations. The average prothrombin times of the remaining chicks, in order of increasing menadione, were 44.1, 30.1, 26.9 and 24.5 seconds, respectively. At the end of 4 weeks none of the chicks fed diets containing sulfaquinoxaline showed normal prothrombin times regardless of the level of menadione.

The requirement for menadione was 500 and 370 μg per kg of diet at two and 4 weeks, respectively. The requirement in the presence of sulfaquinoxaline was 22,580 and 2,680 μg per kg of diet at two and 4 weeks, respectively. The latter calculations were made assuming that the base lines of the two groups would have been the same at the respective ages had sufficient menadione been supplied.

Experiment 4 was conducted to restudy the effect of sulfaquinoxaline on the requirement of the chick for menadione, using larger quantities of menadione in the diet in the presence of this drug. The group of chicks used to establish the reference requirement was fed diets containing 76, 152, 305 and 610 μg of menadione per kg. The levels of menadione in diets with sulfaquinoxaline were 200, 400, 800, 1600, 3200 and 6400 μg per kg.

The results are given in table 2. At two weeks the highest levels of menadione did not completely prevent the increased blood clotting time caused by sulfaquinoxaline. At 4 weeks, however, the two highest levels fed in diets with sulfaquinoxaline had over-

TABLE 2

Effect of sulfaquinoxaline on the menadione requirement of the chick (exp. 4)

Menadione	Av. weight 4 weeks	Feed/gain	Prothrombin time	
			2 weeks	4 weeks
μg/kg diet	*gm*		*seconds*	*seconds*
		Basal diet		
76	465	1.79	26.9	28.7
152	481	1.79	23.7	24.2
305	502	1.71	20.4	22.3
610	471	1.66	19.3	20.8
		Basal diet + sulfaquinoxaline		
200	—	—	83.1	—
400	440	1.84	45.7	33.0
800	397	1.91	36.3	24.5
1600	431	1.72	25.7	22.4
3200	431	1.78	24.3	20.9
6400	451	1.66	21.1	20.4

come the hypoprothrombinemia caused by this drug.

The requirement for menadione was 445 and 460 μg per kg of diet at two and 4 weeks, respectively. The requirement in the presence of sulfaquinoxaline was 8210 and 2330 μg per kg of diet at two and 4 weeks, respectively. The two-week requirement was based on the assumption that the base line for the two groups at two weeks would have been the same had sufficient menadione been supplied in the diet. The base lines for the two groups were the same at 4 weeks.

The regression lines in experiment 4 at 4 weeks in the presence and absence of sulfaquinoxaline are shown in figure 1, and were essentially parallel. The regression lines representing these two groups at two weeks, and those for the two and 4 weeks in experiment 3 were also parallel.

Experiment 5 was conducted to determine the degree to which sulfaquinoxaline increased the MSB requirement of the chick. The reference requirement was established by feeding diets containing 48, 106, 234, 800 and 1600 μg of MSB per kg. The diets with sulfaquinoxaline contained 100, 200, 400, 800, 1600, 3200 and 6400 μg of MSB per kg.

The results are given in table 3. No prothrombin determinations were made at 4 weeks on the chicks fed the diet with sulfaquinoxaline and 100 μg of MSB because of high mortality due primarily to bleeding following the two-week prothrombin determinations.

The requirement for MSB at two and 4 weeks was 635 and 430 μg per kg of diet. At 4 weeks a base line was established for the group of chicks fed the diets containing sulfaquinoxaline and MSB, but in contrast to the experiments with vitamin K_1 and menadione, this base line was not the same as that of the chicks not fed sulfaquinoxaline.

The regression lines for the two groups were parallel at two and 4 weeks. Assuming that the base lines would have been the same at two weeks had sufficient MSB been supplied, the requirement was 13,620 μg per kg of diet. The requirement at 4 weeks was estimated by extrapolating the regression line representing the group fed diets with sulfaquinoxaline to the normal base line. Under these conditions the requirement at 4 weeks was 2680 μg per kg of diet.

The regression lines representing the groups of chicks with and without sulfaquinoxaline are given in figure 1. The regression lines were found to be parallel, the same as those obtained when the data of experiments 2 and 4 were plotted. This indicates a similar pattern of response of all three forms of vitamin K to sulfaquinoxaline. The evident difference was the

TABLE 3

Effect of sulfaquinoxaline on the menadione sodium bisulfite (MSB) requirement of the chick (exp. 5)

MSB	Av. weight 4 weeks	Feed/gain	Prothrombin time	
			2 weeks	4 weeks
μg/kg diet	*gm*		*seconds*	*seconds*
		Basal diet		
48	573	1.68	30.7	34.3
106	482	1.78	26.1	26.1
234	577	1.61	22.6	20.5
800	501	1.76	20.6	17.5
1600	527	1.81	18.9	18.2
		Basal diet + sulfaquinoxaline		
100	—	—	74.9	—
200	469	1.75	52.4	35.7
400	462	1.76	34.8	27.5
800	455	1.74	31.0	26.8
1600	468	1.76	29.7	21.6
3200	458	1.84	24.6	20.4
6400	502	1.59	22.7	21.7

TABLE 4

Comparative abilities of vitamin K_1, menadione (M) and menadione sodium bisulfite (MSB) in overcoming the hypoprothrombinemia of sulfaquinoxaline (SQ) (exp. 6)

Treatment	Av. weight 4 weeks	Feed/gain	Prothrombin time		
			28 days	31 days	Average
µg/kg diet	*gm*		*seconds*	*seconds*	*seconds*
Vitamin K_1					
720	489	1.68	21.1	19.4	19.7
1440	475	1.67	19.9	18.5	
4000+SQ	426	1.72	21.7	19.3	20.3
8000+SQ	420	1.71	21.4	18.8	
Menadione					
610	502	1.66	21.3	19.0	20.2
1220	507	1.72	19.8	19.9	
3200+SQ	406	1.85	21.3	19.6	20.4
6400+SQ	420	1.68	21.2	19.6	
MSB					
800	487	1.74	20.7	18.7	20.1
1600	473	1.69	21.9	19.2	
3200+SQ	432	1.76	23.1	21.5	22.0
6400+SQ	419	1.68	22.6	20.8	

Analysis of variance

Source of variation	Degrees of freedom	Variance ratio	P
Treatment	11	8.77	< 0.001
SQ	1	34.17	< 0.001
Source of K	2	15.70	< 0.001
MSB vs. (K_1+M)	1	30.39	< 0.001
K_1 vs. M	1	1.00	> 0.20
SQ × source	2	7.53	< 0.001
(MSB vs. (K_1+M)) × SQ	1	14.96	< 0.001
(K_1 vs. M) × SQ	1	< 1.00	> 0.20
Remainder	6	2.65	< 0.025
Age	1	136.3	< 0.001
Age × treatment	11	< 1.00	> 0.20
Error	216	1.63[1]	

[1] Error mean square.

failure of MSB to overcome completely the effect of sulfaquinoxaline.

Experiment 6 was conducted to restudy the effect of high levels of vitamin K_1, menadione and MSB in overcoming the hypoprothrombinemia of sulfaquinoxaline. Since, in experiments 2, 4 and 5, at 4 weeks the base lines for the normal chicks and chicks fed diets with sulfaquinoxaline were the same when sufficient vitamin K_1 or menadione, but not MSB, were included in the diet, it was desirable to establish whether this was a chance occurrence.

The base line was formed with diets containing 720 and 1440 µg of vitamin K_1 per kg, 610 and 1220 µg of menadione and 800 and 1600 µg of MSB. The levels fed in diets containing sulfaquinoxaline were 4000 and 8000 µg of vitamin K_1 per kg, 3200 and 6400 µg of menadione and MSB. These levels of vitamin K_1, menadione and menadione sodium bisulfite were the same as used in experiments 1 to 5 to establish the effect of sulfaquinoxaline on the vitamin K requirement of the chick.

The results of experiment 6 and the statistical analysis are presented in table 4. Prothrombin determinations were made at 28 days, and in order to have a check on the results obtained, at 31 days. The determinations at both times were made on the same chicks. The 28- and 31-day data were combined and individual determinations used to establish, by analysis of variance, whether a difference was obtained in the prothrombin times of chicks fed diets containing vitamin K_1, menadione or MSB in diets with and without sulfaquin-

oxaline. No difference was found in the prothrombin times of chicks as a result of the form of the vitamin used when sulfaquinoxaline was omitted from the diet. The prothrombin times of chicks fed diets containing sulfaquinoxaline with adequate vitamin K_1 or menadione were not different from the prothrombin times of chicks receiving diets without this drug. The difference in the prothrombin times of chicks fed sulfaquinoxaline and MSB were significantly higher ($P < 0.001$) than those of the normal chicks. This confirmed the observations in experiment 5 that MSB did not overcome completely the hypoprothrombinemia of sulfaquinoxaline.

These studies with sulfaquinoxaline showed that age had an effect on the response of the chick to the various forms of vitamin K. None of the forms of vitamin K studied was able to overcome the effect of sulfaquinoxaline at two or three weeks at the levels fed. The results obtained at 4 weeks were markedly different from the two- or three-week data obtained in these experiments. A summary of the 4-week results is given in table 5.

The results at 4 weeks show no great difference in the ability of any form of this vitamin to overcome the effects of the drug when expressed on a weight basis. Expressed as the percentage by which the requirement of each form is increased by this drug, the differences still were not very great. When the results were expressed on a molar basis, then vitamin K_1 was slightly more effective than MSB and about 2.5 times as effective as menadione.

The amounts of sulfaquinoxaline inactivated by vitamin K_1, menadione and MSB at 4 weeks are also presented (table 5). The results show that vitamin K_1 was the most potent source of the vitamin studied in overcoming the hypoprothrombinemia of sulfaquinoxaline and that menadione was the least active. The response was much greater at 4 weeks than at two or three weeks, but the relative response was the same. It should be kept in mind, however, that menadione but not MSB completely counteracted the effects of sulfaquinoxaline at 4 weeks. On a molar comparison, menadione and MSB were approximately 40 and 70% as effective as vitamin K_1 in counteracting sulfaquinoxaline. Griminger ('57) reported that one mole of menadione could overcome the effects of approximately 800 moles of sulfaquinoxaline.

In these studies, the results obtained at two weeks were not as consistent as those obtained at 4 weeks. Almquist and Klose ('39) reported that the blood clotting time of vitamin K-deficient chicks was highest at two weeks, after which there was a decline. The possibility exists that in the young chick the blood clotting mechanism has not fully developed and is more susceptible to vitamin K inhibitors than it is at later periods.

It is interesting to compare these results with those obtained by investigators working with dicumarol. Much of the work dealing with this drug has been reviewed by Dam ('48) and indicated that vitamin K_1 was more effective than menadione in overcoming the hypoprothrombinemia caused by this drug. Later, Geill et al. ('54) and Gamble et al. ('55) reported that vitamin K_1 was more effective than menadione in overcoming prolonged blood clotting time induced by dicumarol.

TABLE 5

Effect of sulfaquinoxaline (SQ) on the vitamin K_1, menadione and menadione sodium bisulfite (MSB) requirement of the chick at 4 weeks

Exp. no.	Form of vitamin	Requirement/kg of diet				Increase	SQ inhibited
		No SQ		+ SQ			
		μg	*μmoles*	*μg*	*μmoles*	%	*μmoles*
1	Vitamin K_1	385	0.85	2590	5.75	676	675
2	Vitamin K_1	700	1.55	2750	6.09	393	734
3	Menadione	370	2.14	2680	15.60	729	248
4	Menadione	460	2.67	2330	13.57	508	206
5	MSB	430	1.30	2680	8.13	625	488

CONCLUSIONS

The effect of sulfaquinoxaline on the vitamin K requirement of the chick varied with age. High levels of vitamin K_1, menadione or menadione sodium bisulfite, USP, (MSB) did not overcome the hypoprothrombinemia caused by sulfaquinoxaline at two or three weeks. At 4 weeks vitamin K_1 and menadione prevented this effect. MSB appeared to correct this effect to a certain point beyond which higher levels had no effect.

Although the response of the chick was less at two or three weeks than at 4 weeks, the relative response between these three forms was approximately the same. Rating the response of the chick to vitamin K_1 as 100, menadione was found to be only 40% as effective on a molar basis and MSB was 70% as effective. Thus, vitamin K_1 was more effective than either menadione or menadione sodium bisulfite in overcoming the toxic effects of sulfaquinoxaline. At 4 weeks of age, 0.1% of sulfaquinoxaline in the diet increased the vitamin K_1 requirement 4 to 7 times that found to be required when this drug was omitted from the diet.

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Influence of Heat on the Digestibility of Meat Proteins[1]

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The effect of heat on the nutritive value of proteins has not been clearly established. With respect to proteins of animal source, the manner of heating appears to determine whether a loss of nutritive value occurs. With certain plant proteins, moderate heat has been shown to enhance the food value, whereas more severe treatment lowers the nutritive value. Again, there is evidence that actual destruction of essential amino acids from the protein occurs during heating, whereas other experimental data indicate that reducing sugars combine with amino groups of the protein to yield products largely resistant to the action of proteolytic enzymes.

The last view mentioned has received support from a series of studies, carried out both with intact animals and *in vitro* with crystalline enzymes, in which the effect of heat on the proteins of milk was examined. The evidence strongly suggests that when milk proteins are dry-heated in the presence of reducing sugars, glyconyl peptides are formed which are hydrolyzed only with difficulty by the digestive proteases, with the result that growth is retarded, nitrogen balance becomes negative, and the *in vitro* liberation of amino nitrogen is inhibited. The presence of water in minimal amounts appears to prevent the sugar-peptide formation during heating.

A review of the literature shows that, properly, many of these studies have been carried out on purified proteins; this emphasis, in turn, has largely limited the scope of study to the plant and milk proteins. On the other hand, the importance of meat in the national dietary justifies adequate investigation of the effect of heat on the nutritive value of meat protein. The present comparative study was carried out on several kinds of meat with particular attention to the influence of carbohydrate, water and pH value upon the effect of heat on the *in vitro* digestibility of the meat proteins. Beef (skeletal and cardiac), pork, chicken, scallop and horse muscles were examined.

EXPERIMENTAL

Preparation of samples. Samples of low-fat meat were finely ground and spread out to dry on paper in a cold room at 10°C. Drying was facilitated by the continuous use of a fan for periods varying from 8 to 15 days and the paper was replaced repeatedly to remove excess fat. After the initial drying period, the meat was ground in a Wiley mill and the drying process continued in the cold for an additional two to 13 days. Following this the samples were reground in the mill, packaged and stored in a refrigerator.

Approximately 3.5 kg of sample were obtained from the original 15 pounds of fresh chicken breast. All other meat samples were purchased in a rough-ground form with the exception of scallops which were obtained in a fresh-frozen state. Heart muscle was obtained from fresh beef heart from which all fat was carefully dissected. The samples of pork muscle were the most difficult to prepare due to the inherently high fat content. Scallops developed a glue-like texture upon drying and required repeated handling to allow efficient desiccation. At the end of the drying period, all protein samples were of uniform consistency and were analyzed for total nitrogen, protein, amino nitrogen and soluble carbohydrate (see table 1). Nitrogen was determined by macroKjeldahl, and amino nitrogen by the method of Pope and Stevens

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TABLE 1

Composition of air-dried animal proteins

Protein source	Nitrogen	Protein[1]	Amino nitrogen		Soluble carbohydrate
	%	%	%	*mg/100 mg nitrogen*[2]	%
Beef skeletal muscle	8.9	55.0	0.91	10.2	1.3
Pork muscle	8.0	50.0	0.88	11.0	0.5
Chicken breast muscle	12.7	79.4	2.16	17.0	0.6
Scallops	10.3	64.0	1.96	19.0	1.4
Beef heart muscle	10.3	64.0	1.44	14.0	0.3
Horse meat	12.4	77.5	1.24	10.0	1.0

[1] Calculated on the basis of the contained nitrogen; % nitrogen × 6.25.
[2] Calculated from the percentage amino nitrogen and percentage total nitrogen; used in the addition of glucose to the protein samples such that the soluble carbohydrate and amino nitrogen was the same for all protein samples on the basis of air-dried beef.

TABLE 2

The effect of dry autoclaving (15 pounds pressure for 30 minutes) on various air-dried animal proteins

Protein source	Soluble carbohydrate	Amino nitrogen liberation by pancreatin						
		Hours						
		0	2	4	6	8	24	48
	%	*mg/100 mg nitrogen*						
Beef skeletal muscle								
Unheated	1.3	11.4	20.0	27.3	31.8	35.0	40.0	41.3
Autoclaved	1.2	9.5	17.5	25.0	29.0	34.0	37.5	38.5
Autoclaved	2.5	6.0	14.0	19.0	24.0	27.0	31.5	32.5
Autoclaved	5.0	4.0	11.0	16.5	21.0	24.0	28.5	29.5
Autoclaved	15.0	2.5	9.0	13.5	17.0	19.5	21.0	22.0
Pork muscle								
Unheated	0.5	11.0	21.0	29.5	35.5	39.0	44.5	46.0
Autoclaved	0.5	11.0	22.0	31.0	36.5	40.5	46.5	47.0
Autoclaved	5.0	5.0	15.0	23.5	28.0	31.0	34.5	35.5
Autoclaved	14.0	2.5	11.0	19.0	24.0	28.0	32.0	32.5
Chicken breast muscle								
Unheated	0.6	17.0	31.0	40.0	45.0	49.0	53.0	52.0
Autoclaved	0.6	14.0	27.0	36.0	41.0	45.0	49.0	51.0
Autoclaved	5.0	8.0	20.0	27.0	33.0	36.5	41.5	42.0
Autoclaved	22.0	5.0	15.0	22.0	27.0	31.0	35.0	36.0
Scallops								
Unheated	1.4	19.0	28.0	34.0	38.0	41.5	45.5	47.0
Autoclaved	1.4	22.0	31.0	36.0	40.5	44.0	47.5	49.5
Autoclaved	5.0	10.5	18.0	24.0	28.0	31.0	35.5	36.0
Autoclaved	18.0	3.5	11.0	16.5	22.0	25.0	27.5	28.0
Beef heart muscle[1]								
Unheated	0.3	14.0	23.8	31.0[1]	35.5[1]	—	42.9	44.8
Autoclaved	0.3	12.0	20.8	28.9	33.7	—	40.2	42.0
Autoclaved	5.0	7.0	17.0	22.0	28.0	—	36.0	38.0
Autoclaved	19.0	3.5	11.5	17.5	22.5	—	30.0	32.0
Horse meat								
Unheated	1.0	10.0	19.0	25.0	29.5	32.0	35.5	36.0
Autoclaved	1.0	5.5	15.5	22.0	26.0	28.0	32.0	33.5
Autoclaved	5.0	2.5	10.0	15.0	18.0	22.0	24.0	25.0
Autoclaved	21.5	1.0	7.0	12.5	16.0	19.0	23.0	23.0

[1] The determination of amino nitrogen liberation from beef heart muscle was run at zero, 2, 5, 7, 24 and 48 hours.

('39) as modified by Schroeder et al. ('50). Soluble carbohydrate was determined on 2-gm samples of the dried protein, by extraction with freshly-prepared Folin-Wu tungstic acid solution in a mechanical shaker for 30 minutes. Aliquots of the filtrate were analyzed for soluble carbohydrate by the Folin-Wu sugar method and read on a Bausch and Lomb photoelectric colorimeter. No attempt was made to determine reducing noncarbohydrate compounds which may have been present; soluble carbohydrate was regarded as glucose.

Treatment of the samples. The original protein sample with its contained carbohydrate was heated by "dry-autoclaving" at 15 pounds pressure for 30 minutes, with the unheated sample serving as a control. In addition, glucose, to yield various concentrations, was added to other samples before autoclaving. The final concentration of glucose in the air-dried meat samples is shown in table 2. The maximum glucose added to these proteins was so calculated that the relative concentration of soluble carbohydrate and amino nitrogen was the same as that for air-dried beef containing a glucose concentration of 15% (table 2).

After dry-autoclaving, the *in vitro* liberation of amino nitrogen from the heated and the control samples was studied; in all cases the substrate concentration was calculated on the basis of its nitrogen content for the preparation used. All samples contained 200 mg of protein nitrogen to which was added 200 mg of pancreatin, USP, in 100 ml of phosphate buffer, pH 8.0. At zero time 10-ml aliquots were taken and the remainder incubated at 37°C; samples were removed at 2, 4, 6, 8, 24, and 48 hours for the determination of amino nitrogen. The effect of dry-autoclaving upon the protein substances with their various carbohydrate content is shown in table 2.

In addition to dry-autoclaving, the meat was "reconstituted" with water. Beef was reconstituted to its original 18% protein as found in the low-fat raw beef used. To all other protein sources used, sufficient water was added so that the samples contained the equivalent of beef protein nitrogen, namely 18% protein. This adjustment was made so that the relative concentrations of soluble carbohydrate and amino nitrogen were identical for all meat samples used. This permitted a more equitable comparison of the results of autoclaving the proteins from the different meat sources. In all cases, after reconstitution, the soluble carbohydrate level was the same as in the original protein as well as at the 5% level. The amino nitrogen liberation by pancreatin in the unheated and heated samples of reconstituted meat was studied as before. The results are shown in table 3.

A third series of experiments was carried out in which the effect of autoclaving the various reconstituted meats (18% protein) containing 5% of soluble carbohydrate at various pH levels was observed. The reconstitution and added carbohydrate were calculated as defined previously and the samples heated at pH 7.0, 9.0 and 11.0 in phosphate buffer solutions. Again the release of amino nitrogen was studied as before; the results are shown in table 4. The liberation of amino nitrogen from the various samples is presented graphically for beef protein in figure 1; all other protein samples showed similar results.

RESULTS AND DISCUSSION

The results of the present study on meat proteins which were heated under various environmental conditions are, in general, in good agreement with those observed in our extensive studies on milk proteins. The soluble carbohydrate content in the original air-dried samples of the various animal proteins shows little variation. Distinct differences, however, in both the total nitrogen and amino nitrogen were observed (table 1). Hence, in all reconstitution studies, beef protein was adopted as standard, such that all animal proteins when reconstituted would contain the same relative amounts of nitrogen and soluble carbohydrate.

The results of dry-autoclaving the various air-dried meat proteins at different carbohydrate levels are shown in table 2. Analysis of these data shows that such treatment, even at minimum carbohydrate levels (original levels), caused a decrease in digestibility for all meat proteins with the exception of pork muscle and scallops. As the level of glucose was increased,

TABLE 3

The effect of autoclaving (15 pounds pressure for 30 minutes) on various reconstituted animal proteins[1]

Protein source	Soluble carbohydrate	Amino nitrogen liberation by pancreatin						
		Hours						
		0	2	4	6	8	24	48
	%	*mg/100 mg nitrogen*						
Beef skeletal muscle								
Unheated	1.3	14.0	22.5	29.5	34.0	37.0	41.0	42.0
Autoclaved	1.3	13.5	23.0	30.0	36.0	39.0	44.0	43.8
Unheated	5.0	12.5	22.0	29.0	35.0	38.0	42.5	44.0
Autoclaved	5.0	8.5	17.5	24.0	29.0	32.5	37.0	38.0
Pork muscle								
Unheated	0.5	15.0	26.0	34.5	40.0	44.0	50.0	52.0
Autoclaved	0.5	12.0	24.5	33.0	39.0	42.0	49.0	51.0
Unheated	5.0	12.0	23.0	32.0	37.0	40.0	47.5	50.0
Autoclaved	5.0	11.0	21.5	30.0	35.5	39.5	45.5	47.0
Chicken breast muscle								
Unheated	0.6	18.0	29.5	38.0	43.5	47.0	51.0	52.0
Autoclaved	0.6	19.0	32.0	41.0	46.0	50.0	53.0	54.0
Unheated	5.0	15.0	28.0	37.0	44.0	50.0	54.0	55.5
Autoclaved	5.0	13.0	26.0	34.0	40.0	44.0	48.0	49.5
Scallops								
Unheated	1.4	21.0	29.5	35.5	39.0	42.5	47.5	47.5
Autoclaved	1.4	20.0	32.0	38.0	42.5	45.0	49.5	50.5
Unheated	5.0	17.0	24.5	30.5	34.5	37.5	42.0	45.0
Autoclaved	5.0	18.0	26.0	32.0	37.0	41.0	43.0	44.0
Beef heart muscle[2]								
Unheated	0.3	13.0	31.5	37.2[2]	41.5[2]	—	46.0	47.0
Autoclaved	0.3	10.5	28.0	34.3	38.4	—	43.9	45.0
Unheated	5.0	11.0	27.8	35.9	39.3	—	45.0	46.0
Autoclaved	5.0	9.5	26.7	34.0	37.8	—	42.5	43.5
Horse meat								
Unheated	1.0	9.5	18.0	24.0	28.0	30.5	33.5	35.0
Autoclaved	1.0	13.0	23.0	28.5	34.0	36.0	40.5	40.5
Unheated	5.0	11.0	20.5	27.0	31.0	34.0	37.5	38.0
Autoclaved	5.0	11.0	22.0	28.0	32.5	35.5	39.0	39.5

[1] All animal proteins were reconstituted to 18% protein with water; this reconstitution yielded the same protein concentration as the original beef skeletal muscle and permitted the relative ratios of carbohydrate to protein to remain constant for all protein sources.

[2] The determination of amino nitrogen liberation from beef heart muscle was run at zero, 2, 5, 7, 24 and 48 hours.

however, there was a progressive decrease in digestibility as measured by liberated amino nitrogen in all the samples used when the mixtures were dry-autoclaved. Such results are to be expected in view of earlier observations from this laboratory and others, inasmuch as increase in carbohydrate content augments the chance for interaction between sugars and amino compounds.

In general, if the system is reconstituted with water to 18% protein prior to autoclaving, the decrease in digestibility due to increasing glucose concentrations is still observed, but to a lesser extent than without reconstitution (table 3). At the original carbohydrate levels, however, the reconstituted protein shows a slight increase in digestibility after autoclaving the proteins used here, with the exception of pork muscle and beef heart muscle where slight decreases in amino nitrogen liberation are seen. When the carbohydrate level is increased to 5%, autoclaving causes a decrease in digestibility to some extent in all proteins with the exception of horse meat; a slight increase was observed in this case. The increased sugar content plays a major role, but when a comparison at the 5% sugar level is made between the

TABLE 4

The effect of autoclaving (15 pounds pressure for 30 minutes) on various reconstituted animal proteins[1] containing 5% of soluble carbohydrate at different pH levels

Protein source		Amino nitrogen liberation by pancreatin						
		Hours						
		0	2	4	6	8	24	48
	pH	*mg/100 mg nitrogen*						
Beef skeletal muscle	7.0	8.0	16.5	23.0	28.0	31.5	36.0	37.0
	9.0	5.0	14.5	20.0	24.5	28.0	32.0	33.0
	11.0	4.0	11.0	16.0	19.2	22.0	25.0	26.0
Pork muscle	7.0	10.5	20.0	28.0	34.0	37.0	43.5	44.5
	9.0	7.0	18.0	26.0	31.5	35.0	39.5	40.0
	11.0	7.0	16.0	24.0	29.5	33.0	37.0	38.0
Chicken breast muscle	7.0	12.0	24.0	33.0	39.0	43.0	47.0	48.5
	9.0	9.5	21.0	28.5	34.5	38.5	43.0	43.5
	11.0	7.0	18.5	26.0	31.5	35.0	40.0	41.0
Scallops	7.0	15.5	23.0	29.5	33.0	36.5	41.0	42.0
	9.0	13.0	20.0	25.5	29.5	33.0	38.5	39.0
	11.0	5.0	14.0	18.0	23.0	26.0	29.0	30.0
Beef heart muscle[2]	7.0	9.0	25.0	32.3[2]	36.2[2]	—	42.0	43.0
	9.0	5.0	22.0	29.0	33.0	—	39.5	40.5
	11.0	3.0	18.5	25.8	30.5	—	37.8	39.0
Horse meat	7.0	6.5	13.5	20.0	23.0	26.0	29.5	30.5
	9.0	4.0	11.0	16.5	20.0	22.5	26.0	26.5
	11.0	1.0	6.0	12.0	15.5	18.0	21.5	22.0

[1] All animal proteins were reconstituted with water to 18% protein as before (see table 3).
[2] The determination of amino nitrogen liberation from beef heart muscle was run at zero, 2, 5, 7, 24 and 48 hours.

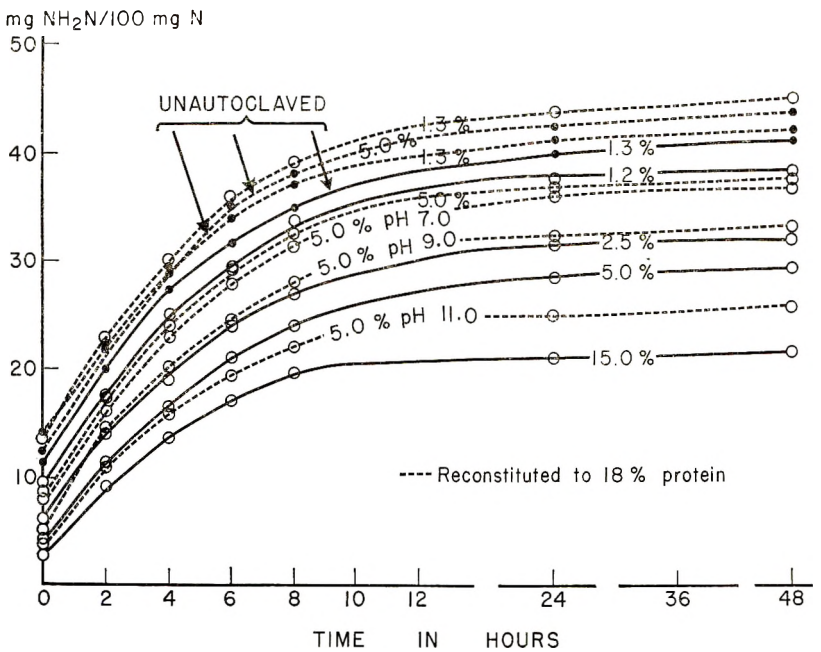

Fig. 1 The liberation of amino nitrogen per 100 mg of nitrogen from air-dried beef skeletal muscle by pancreatin.

original air-dried and the reconstituted proteins, this effect is largely minimized. The "protective action" of the added water varied; when the dry-autoclaved protein and the reconstituted protein (both containing 5% of glucose) were compared, the minimum increased digestion occurred with heart muscle protein (14.5%), whereas the maximum was observed in horse meat (58%). These results warrant the conclusion that any ordinary heating process of meat protein improves the nutritional quality of the protein.

The results of autoclaving the reconstituted proteins in the presence of 5% of soluble carbohydrate at various pH levels may be seen in table 4. With the exception of horse meat, the liberation of amino nitrogen at pH 7.0 is approximately the same as for that in an unbuffered aqueous solution. As is to be expected, as the pH of the reconstituted mixture is increased before autoclaving, the resultant digestibility is decreased. In some instances, the decrease is more dramatic than in others; this response is greatest in the case of scallops, whereas in that of heart muscle the effect was minimum. It must be remembered, however, that all proteins were reconstituted to the same level of nitrogen and the relative ratio between the contained protein and carbohydrate was constant. The high percentage of amino nitrogen found in scallops may account for this greater interaction although this suggestion is not borne out in other samples.

The results of this study are well summarized in figure 1, which graphically represents for beef protein the results of the different types of environmental variation used in the present study. Similar, and in some cases almost identical, results were observed for the other meats used. Comparison of the data on the various meats (tables 2, 3 and 4) with those presented graphically for beef, shows that in most cases the rate of liberation of amino acids by pancreatin from the variously treated (mild) meats is almost the same. This indicates that the major difference between the treated meats lies in the extent of liberation of amino nitrogen. It appears, therefore, that no appreciable destruction of amino acids occurs, but rather, the formation of such linkages resistant to proteolysis by pancreatin. On the other hand, the exception to this observation that the rate of amino nitrogen liberation is relatively uniform, occurs only in those meats which had been drastically treated, i.e., autoclaved at a more alkaline pH and those which were dry-autoclaved with high concentrations of carbohydrates. As shown graphically for beef, the slopes of the amino nitrogen liberation curves are quite different for meats treated in this manner. In these cases, the extent of liberation as well as the rate of amino nitrogen liberation decreased. In these instances some destruction of amino acids could conceivably occur.

In an early study, Scheunert and Bischoff ('30) showed that boiled and autoclaved (1 atm.) meat was superior for growth and reproduction in rats to meat autoclaved at 4 atm. Their investigation called attention to the diminishing nutritional returns caused by overheating of protein. This point was emphasized by Morgan and Kern ('34) who fed meat raw, boiled and autoclaved (7 minutes at 15 pounds, and 1 hour at 15 pounds) to rats and observed the nitrogen balance and protein efficiency. Beef liver heated at 120°C for 72 hours dropped in biologic efficiency as a result of decreased digestibility (Seegers and Mattill, '35), whereas heating at 100°C for two weeks produced little loss of biologic value. The canning process reduced slightly the nutritive value of cured pork muscle, whereas frying seemed to increase the value of fresh pork (Poling et al., '44). Although the decrease in nutritive value of heated proteins has been attributed to a possible destruction of amino acids, Beuk and associates ('48) failed to demonstrate any destruction of the indispensable amino acids in fresh, fresh-autoclaved, cured, and cured-autoclaved pork. In comprehensive comparisons between canned samples of meat and fish products and the raw materials, no loss of any indispensable amino acids was observed (Dunn et al., '49; Nielands et al., '49). Again, roasting of beef for 5 hours at 149°C produced no deleterious effect on the biologic value of the meat protein according to Mitchell et al. ('49). On the basis of variation in food efficiency with

rats, McBride et al. ('51) observed little adverse influence of heat, using fried-bacon protein. Pressure cooking and braising at ordinary cooking temperatures did not adversely affect the nutritive value of the meat protein as measured by the nitrogen balance, nitrogen efficiency of utilization, nitrogen storage and body weight gain in growing rats (Clark et al., '55). Wheeler and Morgan ('58) fed controlled amounts of raw fresh pork and of pork autoclaved for periods of 4 to 14 hours at 120°C to adult and growing rats. On the basis of quantitative analysis for amino acids in portal blood at intervals after feeding, these investigators concluded that, with heated pork, there was both a delay in the peak of concentration of amino acids and the level attained, as compared with control animals fed raw pork muscle. From the available evidence, it appears that the proteins of meat are not greatly diminished in nutritive value by methods of heating used in ordinary cookery; on the other hand, prolonged high temperatures appear to decrease the overall utilization of meat proteins.

There are inherent experimental difficulties in studying meat as compared with purified proteins and it is to be expected, therefore, that more precise delineation of environmental factors regarding the effect of heat on food proteins should have been secured with the purified proteins. In 1948 Patton et al. observed that upon refluxing casein with 5% glucose (mild heating), arginine, histidine and lysine were inactivated. Dry commercial lactalbumin is altered by autoclaving so that *in vivo* digestibility by the rat is decreased and growth is inhibited (Davis et al., '49). However, similar heat treatment of extracted lactalbumin did not prevent the maintenance of nitrogen balance in dogs (Mader et al., '49). Again, autoclaving extracted lactalbumin in the presence of reducing sugars (lactose, maltose, glucose, xylose and fructose) resulted in distinctly negative nitrogen balances when the heated lactalbumin was the source of protein in the diet (Schroeder et al., '51). The foregoing studies suggest that, in the presence of reducing sugars, heat brings about a deleterious change in food proteins which lowers their nutritive value through decreased digestibility.

The failure of autoclaving of fluid milk to lower the nutritive value of the milk proteins (Schroeder et al., '53a) led to a further study of the influence of water on heat damage to protein (Schroeder et al., '53b). Fat-free milk solids containing 35% protein were autoclaved as such, as well as after reconstitution to 24.5, 17.5, 10.5 and 3.5% protein. *In vitro* digestibility experiments with crystalline trypsin or chymotrypsin showed that in every instance where water had been added prior to autoclaving, the rate of amino acid liberation was enhanced over that of the unheated reconstituted milk. The *in vitro* observations were confirmed by nitrogen balance studies on dogs. The same influence of water on the effect of autoclaving was demonstrated with glucose:amino acids mixtures and glucose:synthetic peptide mixtures (Schroeder et al., '55a, '55b). It has been postulated that when the nutritive value of a protein is diminished by heating with reducing sugars, an amino acid-sugar combination is formed with a concomitant drop in the free amino nitrogen. Schroeder et al. ('55a) have shown that heat brings about this change readily when the pH of the system (glucose:dipeptide) is buffered above 6.0, but not at a more acid reaction. The various mechanisms which have been proposed for the interaction between carbohydrates and amino compounds have been reviewed extensively by Ellis ('59).

In general, one may conclude that the proteins of meat react in a manner similar to purified proteins and the proteins of milk. In order to elicit major differences in amino acid liberation from meat proteins, it was found necessary to alter drastically the environmental conditions by increasing the concentration of carbohydrate or the pH much beyond the limit which may conceivably be used in cookery. Hence, ordinary cooking processes do not have an unfavorable effect on the nutritional value of the protein, but rather may cause its improvement to some degree.

SUMMARY

Air-dried samples of beef (skeletal and cardiac), pork, chicken, scallop and horse

muscles were prepared and analyzed for nitrogen, amino nitrogen and soluble carbohydrate.

The original meat samples, as well as those to which various concentrations of glucose were added, were subjected to dry-autoclaving. In addition, samples were reconstituted with water and, in other cases, were buffered at various pH levels before autoclaving. The digestibility of all samples was evaluated by liberation of amino nitrogen by pancreatin.

Digestibility decreased with increasing carbohydrate concentration upon dry-autoclaving. When reconstituted, such decreases were largely eliminated and the proteins showed increased susceptibility to proteolysis. Increasing pH levels fostered some interaction between carbohydrates and proteins as evidenced by decreased liberation of amino nitrogen.

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Mineral Deficiencies of Milk and Congenital Malformations in the Rat[1]

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The relation of nutrition to experimental teratology has been studied extensively in recent years, but the effects of trace mineral deficiencies have received little attention. (Hogan, '53; Kalter and Warkany, '59).

The newborn of manganese-deficient dams usually die soon after birth but the survivors exhibit ataxia, incoordination and loss of equilibrium (Daniels and Everson, '35; Shils and McCollum, '43; Hurley et al., '58). Recent observations of Hurley et al., ('60) suggest that the ataxia of manganese deficient offspring results from an anomalous ossification of the inner ear. The offspring of manganese-deficient guinea pigs also show ataxic symptoms at birth (Everson et al., '59). Barnes et al., ('41) presented limited evidence that the offspring of manganese-deficient rats had abnormally short tibiae.

Although iron-deficiency anemia has been extensively investigated, the effect of iron deficiency and the resulting anemia on the developing embryo has not been tested in experimental animals. From human studies by Sisson and Lund ('58), these workers concluded that the infants of anemic mothers usually share their mothers' anemia. Experimental data relating copper deficiency and embryonic development are also meager. However, an enzootic ataxia characterized by cerebral demyelination was observed in lambs born to ewes that consumed large quantities of seaweed (Palsson and Grimsson, '53). The disorder was largely prevented by copper supplementation of the ewes during the gestation period.

Whole milk affords one of the simplest basal diets for study of iron, copper and manganese deficiency in the rat, but its use in most laboratories has not adequately supported reproduction and lactation when supplemented with the deficient minerals (Waddell et al., '31; Richardson and Hogan, '40). On the other hand, Daniels and Everson ('35) and Hurley et al., ('58) have been relatively successful in maintaining reproduction and lactation in rats fed fluid, whole-milk diets.

The results presented in this paper show the teratological effects of mild to moderately severe maternal deficiencies of iron, manganese and copper in the newborn rat.

EXPERIMENTAL

The control diet was composed of the following: (in grams) dried whole milk,[2] 990; glucose hydrate (as vitamin carrier), 10; and (in milligrams) iron as $FePO_4 \cdot 4H_2O$, 200; copper as $CuSO_4 \cdot 5H_2O$, 16; manganese as $MnSO_4 \cdot H_2O$, 240; iodine as KI, 18; cobalt as $CoCl_2 \cdot 6H_2O$, 0.6; α-tocopheryl acetate, 30; menadione, 10; thiamine·HCl, 16; riboflavin, 16; pyridoxine·HCl, 16; Ca pantothenate, 40; biotin, 0.4; folacin, 5; cyanocobalamin, 0.05; and vitamin A, 20,000 I.U. and vitamin D, 2850 I.U.[3] The diets deficient in iron, copper or manganese were prepared by omitting the respective mineral supplements. By analysis the unsupplemented milk contained on the average 1.0 ppm of manganese and 0.5 ppm of copper. The dry diet was fed

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[2] Parlac, The Borden Company, New York.
[3] The authors gratefully acknowledge gifts of vitamins from Merck Sharp and Dohme, the American Cyanamid Company and Hoffman-LaRoche, Inc.

ad libitum and distilled water was supplied in glass bottles.

Approximately 25 weanling female rats of the Wistar strain were allocated to each of the 4 diets described. Groups of 5 were placed in suspended, galvanized cages coated with an epoxy resin. The animals were weighed weekly and when they reached maturity, 170 to 180 gm and about 16 to 18 weeks of age, stock colony males were introduced into the cages. When pregnant, the females were separated and allowed to litter on paper or clean wood shavings. The newborn were examined, as previously described (Grainger et al., '54), for gross abnormalities such as hydrocephalus, eye defects and cleft palate and in most cases were cleared and stained for examination of the skeleton. A small proportion of the litters were allowed to remain with their mothers to determine the viability of the offspring and the lactation performance of the mothers. The dams were returned to the breeding cage immediately after each litter was removed, and allowed to reproduce continuously for about one year.

The packed red cell volume was determined periodically on the adult rats by use of Van Allen hematocrit tubes and hemoglobin was determined on randomly selected newborn by the cyanmethemoglobin method (Drabkin and Austin, '35). The coagulation time of blood from newborn rats was determined by the capillary tube method (Farris and Griffith, '49). A capillary tube was drawn to a fine point and used to obtain blood directly from the heart. Immediately after blood was drawn by cardiac puncture, the time was noted and portions of the tube were broken off at 15-second intervals until coagulation occurred.

Animals saved for histological examination were fixed in Baker's fixative. Portions of organs were embedded in paraffin, sectioned at 7 μ, and stained with hematoxylin and eosin in the routine manner (Armed Forces Institute of Pathology, '60).

RESULTS

Numerous investigators have observed that the highest incidence of congenital malformations caused by nutritional deficiency occurs with diets only moderately deficient. Severe deficiency usually results in death and resorption of the embryo and consequently teratology is not observed. For this reason no attempt was made in this investigation to develop diets extremely low in the respective minerals. During the first 4 weeks of the diet period, all groups gained at the same rate, 18 gm per week.

The diet low in iron produced a mild anemia which was evident in the dams by the end of the 4th month or earlier. The hematocrit was determined at intervals during the reproduction period and the average values are shown in table 1. At one time during the period, the copper-deficient dams were slightly anemic, but the hematocrit values had returned to normal by the end of the period. This may have been a normal response to this diet and its concentration of copper or it may have resulted from contamination of the diet with copper. It was not possible to analyze each batch of feed over the rather long period of the trial.

Of the 1300 offspring born to dams fed the control diet, about 6% were dead at birth (table 2). Of the 131 allowed to nurse, only 20% were raised to weaning. This low weaning percentage demonstrates clearly the inadequacy of the supple-

TABLE 1

Packed red cell volumes of female rats fed milk diets from weaning through the reproductive period

Omission from control diet	No. months rats received diet			
	4–6	7–9	10–12	13–15
None	47(24)[1]	49(7)	44(12)	47(8)
Iron	37(17)	37(13)	40(12)	40(9)
Manganese	44(14)	49(10)	46(11)	43(10)
Copper	45(14)	47(14)	41(14)	48(13)

[1] Number of observations shown in parentheses.

TABLE 2

Lactation performance of rats fed milk diets and congenital malformations in their offspring

Omission from control diet	No. of litters	Total no. born	Born dead	Weaned at 4 weeks	Abnormalities at birth					
					Hematocrit	Hemoglobin	Hemorrhage	Hydrocephalus	Eye defects	Bone defects
			%	%	%	*gm/100 ml*	%	%	%	%
None	199(30)[1]	1300	6.4	20(131)[2]	38(23)[2]	13.7(38)[2]	0	0.15	3.3	5.5(456)[2]
Iron	115(25)	611	6.9	3(130)	28(13)	9.4(52)	0	0.16	7.4	7.4(430)
Manganese	136(25)	929	8.9	14(289)	33(16)	—	0	0.11	10.0	10.5(408)
Copper	93(26)	574	6.4	9(159)	25(14)	8.8(31)	24	0.15	4.2	12.2(320)

[1] Number of females started on diet.

[2] The number of observations on which the percentage is based is shown in parentheses.

mented, dried-whole-milk diet. Since it was grossly inadequate for the support of lactation, little can be concluded regarding the effects of the deficient diets on lactation except to say that they were all less adequate than the control diet. The control diet supported reproduction adequately and the newborn appeared to be as large and vigorous as offspring from the stock colony. The average hematocrit was 38% and the average hemoglobin, 13.7 gm per 100 ml. The incidence of eye and bone defects was about the same as when using our stock diet, but there was a consistent, though low, incidence of hydrocephalus with all of the milk diets regardless of whether they were deficient in one of the minerals under study. Although the incidence was low, it was far greater than commonly observed when feeding the stock diet or complete, purified diets. The dietary cause of the malformation is obscure at present.

Iron deficiency and teratology. As noted above the omission of iron from the control diet caused a mild anemia in adult rats consuming it and the anemic state was conferred upon the newborn. The iron-deficient offspring were small, pale and almost none survived more than a few days. One of the objectives of studying iron deficiency was to observe the effect of the anemic state of the mother upon teratology in the offspring. Except for the anemia and lack of viability, there was only a slightly higher incidence of gross malformation among the iron-deficient progeny. The percentage of eye defects was higher but the percentage of bone anomalies was not significantly affected.

Manganese deficiency. The omission of manganese did not impair reproduction appreciably, but the lactation performance was inferior to that with the control diet. Since reproduction was not impaired and only a few of the animals reared to weaning showed incoordination, it is clear that a severe deficiency did not exist. However, the incidence of both eye and bone deformities was increased. The nature of the skeletal malformations deserves further comment. Several litters receiving the control and manganese-deficient diets were carefully cleared for observation of the long bones, the sternum and the spinal

TABLE 3

Effect of low manganese diet on skeletal development and malformations

Omission from control diet	No. observed	Average weight	Radius length	Tibia length	Vestigial sternebrae[1]
		gm	*mm*	*mm*	%
None	71(17)[2]	5.0	4.5 ± 0.001[3]	4.1 ± 0.001	0
Manganese	86(14)	4.7	4.0 ± 0.001[4]	3.7 ± 0.001[4]	56

[1] An extra calcification site between the 5th and 6th sternebrae.
[2] Number of litters shown in parentheses.
[3] Standard error of mean.
[4] Statistically different from the control diet ($P < 0.01$).

column. The lengths of the radii and tibiae were measured under a dissecting microscope and the averages are presented in table 3. The bones were about 10% shorter in the manganese-deficient offspring and statistically this difference was highly significant. The birth weights were also about 5% less but the significance of this relationship was not obvious. Another rather striking difference was the occurrence of an extra or vestigial sternebrum in the sternum of a high percentage of the deficient newborn. A higher than normal incidence of fusion of sternal and vertebral segments was also noted.

Copper deficiency. The most striking teratology observed in this study occurred among the offspring of dams that consumed the diet low in copper. Not only were the newborn anemic and nonviable, but about one-fourth of them was afflicted with edema and a characteristic subcutaneous hemorrhage. In fact, the internal blood loss was apparently sufficient in many cases to account for the anemic and bleached appearance of the dead or morbid animals. In addition to the generalized hemorrhage, in many cases there was a characteristic and localized ischemia or bleaching of the extremities such as the feet and tip of the tail. The copper-deficient offspring showed a high incidence of skeletal anomalies and many had abdominal hernia.

The general appearance of a copper-deficient litter is shown in figure 1 and may be compared with controls shown in figure 2. The deficient animals showed widespread and diffuse hemorrhage. A cross section of the upper portion of the rear leg of deficient newborn is shown in figure 3 and may be compared with a control section shown in figure 4. Hemorrhage was most prevalent at the junction of subcutaneous tissue and skeletal muscle, but was also observed within muscle tissue as well as in the area near the epidermis. Occasionally there were small perivascular hemorrhages, but in general the endothelium appeared intact. Nearly all sections of copper-deficient animals showed evidence of edema. Particularly striking was the greatly decreased number of hair follicles in the deficient animals.

The hemorrhagic syndrome which is characteristic of the copper-deficient, newborn rat could be the result of a vessel defect or a coagulation defect, but our observations on coagulation time tend to rule out the latter possibility. The time required for cardiac blood to coagulate averaged 1.6 minutes for 20 rats born to dams fed a control diet and 1.7 minutes for 30 rats fed a copper-deficient diet.

DISCUSSION

The diets used in this investigation were not sufficiently low in the various minerals to affect growth rate or to impair reproduction seriously in the adult animal, but they did cause marked abnormalities in the offspring. In general it appears that the pattern of abnormalities due to mineral deficiencies differs somewhat from that observed in vitamin deficiencies. In our rat colony deficiencies of folacin or vitamin B_{12} result in a high incidence of central nervous system disorders, such as hydrocephalus, and in a somewhat lower incidence of cleft palate and eye abnormalities, (O'Dell et al., '48, '51). Moderate deficiences of iron, manganese and copper, as produced in this study, did not increase the incidence of hydrocephalus and had no effect on palate development. There was an appreciable effect on the eyes, and

both manganese and copper had a marked effect on bone development.

A low incidence of hydrocephalus occurred with all of the milk diets. This is no doubt a dietary effect but the exact cause is unknown. With the addition of the supplements, it seems unlikely that the incidence is due to a vitamin deficiency. Milk, with its high lactose content, is not a normal food for an adult mammal and possibly the lactose itself is detrimental.

In view of the known teratologic effects of anoxia it is unexpected that iron-deficiency anemia in the mothers did not cause a higher incidence of gross malformations. The mild deficiency of manganese had its greatest effect on skeletal development and this was to be expected in view of

Fig. 1 Copper-deficient newborn showing typical diffuse hemorrhage and bleaching or ischemia of extremities.

Fig. 2 Newborn rats from control diet. Note uniformity of color.

Fig. 3 Portion of cross section of thigh from a copper-deficient newborn showing a massive hemorrhage between the subcutaneous tissue and muscle. Note space due to edema and the paucity of hair follicles. Hematoxylin and eosin. × 80.

Fig. 4 Cross section from control newborn in same area as described above. Note large number of hair follicles. Hematoxylin and eosin. × 80.

earlier work on the developing chick embryo (Lyons and Insko, '37; Caskey and Norris, '40). Although copper deficiency in the developing embryo had an effect on bone development, its major effect was on soft tissue. At the present state of knowledge the specific function of copper in prevention of the hemorrhagic tendency is not known, but apparently copper deficiency results in a vessel defect which allows loss of blood into the surrounding tissue. In view of the known effects of copper deficiency on bone development (Follis, '58) and the present observations on the susceptibility of deficient animals to hemorrhage, it seems reasonable that copper has a specific function in connective tissue development. The common occurrence of abdominal hernias in the copper-deficient newborn would also lend support to this hypothesis.

SUMMARY

Female rats were maintained from weaning through the reproductive period using 4 different dried-whole-milk diets. The control diet was supplemented with vitamins and the known deficient minerals, iron, manganese, copper and iodine. Diets low in iron, manganese or copper were prepared by omission of the respective supplement.

Iron deficiency caused a mild anemia in the adult rats. Their offspring were severely anemic, weak and almost entirely nonviable, but otherwise not grossly malformed.

The mild manganese deficiency induced by the whole-milk diet did not seriously impair reproduction but it did produce skeletal anomalies. The long bones were shorter than normal, there were extra sternebrae in the sternum and fusion of the sternal and vertebral segments was common.

The copper-deficient diet did not cause anemia in the adult animals but the offspring were severely anemic and almost entirely nonviable. The deficient newborn was characterized by severe edema, widespread subcutaneous hemorrhage and abdominal hernias.

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Porcine Neonatal Nutrition: Absorption of Unaltered Nonporcine Proteins and Polyvinylpyrrolidone from the Gut of Piglets and the Subsequent Effect on the Maturation of the Serum Protein Profile[1]

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In a previous publication the authors have pointed out that baby pigs are born extremely deficient in serum proteins; lacking γ-globulin, scant albumin and β-globulin, low total serum proteins, and 70 to 80% of the protein migrating in the α-globulin zone (Lecce and Matrone, '60). This immature piglet profile is similar to the serum protein profile of fetal goats and sheep in the first third of the gestation (Barboriak et al., '58a, '58b; Meschia, '55).

Intense serum protein changes, such as a doubling in total serum proteins and marked increase in the β-γ-globulin zone, occur within the first 24 hours of life in piglets nursing the sow. Pigs receiving only 24 hours of colostrum, however, when switched to cow's milk, cannot maintain these changes nor can they continue the serum protein maturation process without delay — resembling in this respect the latency found in colostrum-free piglets fed cow's milk from birth (Lecce and Matrone, '61). Piglets had to nurse the sow from two to 4 days before their metabolic activity apparently was sufficient to sustain the uninterrupted development toward a mature serum profile. Thus, the sow's lacteal secretion seems to influence two different phases in the piglet's serum protein maturation process: (1) the initial one involving the immediate changes occurring in the first 24 hours, and (2) the subsequent phase involving the continuing changes occurring thereafter.

It has been assumed by us and others, using changes in electrophoretic fractions and increases in antibody titers as testing criteria, that the changes in the piglet's neonatal serum protein profile occurring in the first phase of the maturation process resulted from the absorption of proteins unaltered from the gut (Nordbring and Olsson, '57; Rutqvist, '58; Olsson, '59a, '59b; Lecce and Matrone, '60). Mainly, these data depend upon the demonstration of electrophoretic protein fractions or specific antibodies in sow's colostrum and a similar demonstration of an immediate increase of either of these components in the serum of the piglet after having nursed.

Since there is no γ-globulin in neonatal pig serum, evidence for absorption into this fraction readily lends itself to assay by electrophoresis or changes in antibody γ-globulin. Thus, the evidence for the absorption of unaltered γ-globulin is convincing. But minor increases in major fractions already present in neonatal pig serum do not lend themselves to an unequivocal detection by either of the above methods. Therefore, for reasons just stated, information concerning the absorption of other proteins or the specificity of absorption is practically lacking.

It was the purpose of the experiments reported herein (1) to investigate further the absorption of unaltered proteins from the gut of the piglet; (2) to determine the specificity of the absorption mechanism; and (3) to study the influence of diets containing nonporcine proteins on the de-

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velopment of mature serum protein profiles in piglets.

EXPERIMENTAL

Neonatal pigs were fed cow's colostrum, cow's milk fortified with avian eggs, and cow's milk fortified with the nonprotein blood plasma extender, polyvinylpyrrolidone (PVP).[2] The cow's colostrum was chosen because it contains proteins functionally similar to those in sow's colostrum and milk, mainly γ- or immune globulin. Eggs were fed because they probably contain proteins that are functionally different and also far removed phylogenetically from proteins in sow's milk and colostrum. The synthetic plasma extender (PVP) was tested because it is similar to serum proteins with regard to plasma osmotic pressure regulation and molecular size.

To determine the specificity of the absorption phenomenon (which proteins were being absorbed unaltered by the piglet), the highly specific and sensitive protein-analyzing technique of immunoelectrophoresis was used (Grabar and Williams, '53). Agar electrophoresis was used for the detection of absorbed PVP. The effect of these diets on the development of serum protein profiles was evaluated by techniques and criteria previously reported (Lecce and Matrone, '60).

Protein analysis. Three measurements characterized the serum protein profile: (1) relative concentration of individual serum proteins, (2) amount of trichloroacetic acid (TCA) precipitate, and (3) percentage of total serum protein (Lecce and Matrone, '60).

Antisera. Reference antisera to egg white proteins and bovine whey proteins were prepared in mature rabbits. Usually 4 rabbits were injected intravenously a minimum of 16 times over a 6-week period with each protein source. At least a total of 600 mg of protein was given to each rabbit. Antisera showing maximum number of immunoelectrophoretic arcs were pooled and used in later tests.

Immunoelectrophoresis. LKB paper electrophoretic equipment was modified in developing a microimmunoelectrophoretic technique similar to Scheidegger's ('55). Using a template made with two razor blades and two pieces of capillary tubing imbedded in modeling clay, two holes and a center trough were cut in an agar bed. The agar bed, resting on a 75 × 25 mm microscope slide, consisted of 2 ml of 0.85% ionagar[3] in pH 8.6 barbital buffer, ionic strength, 0.05. Usually, 0.002 to 0.004 ml of serum (antigen) was placed in one of the holes, with a like amount of reference antigen in the other hole. These antigens then were separated by electrophoresis for 45 minutes with a voltage drop across the slide of approximately 50 V. After removing the slides from the electrophoresis apparatus, 0.04 ml of reference antiserum was placed in the center trough. The slides were placed horizontally in a closed, moist slide box for 20 to 24 hours. At the end of this time, pronounced arcs of precipitation between antigen and antibody were seen. To make a permanent record, slides were washed 6 times over a 36-hour period with 0.9% NaCl and for 12 hours in distilled water. Slides were rapidly dried by placing them under an infrared lamp and a current of moving air. Staining was accomplished by immersing the slides for 10 minutes in a saturated solution of amido black 10B in methanol, acetic acid, and water (45, 10, 45). Slides were washed for 10-minute intervals in three separate baths of the same methanol-acetic acid-water mixture used in staining.

Agar electrophoresis, carried out under conditions similar to those used for immunoelectrophoresis, was used for the separation of egg proteins, colostral proteins, and for the detection of PVP. Usually, 0.005 ml of fresh egg white diluted 1:2 in saline, 0.002 ml of cow's colostral whey, or 0.015 ml of piglet serum was placed in a 1.6 cm slit running across the slide. After electrophoresis the slides were fixed for an hour in either 2% acetic acid in 80% ethyl alcohol to precipitate the proteins or 8% tannic acid to precipitate the PVP. The slides then were dried and stained as indicated above with immunoelectrophoresis (with the exception of washing those slides fixed with tannic acid

[2] Average molecular weight of 40,000. Antara Chemicals, New York.

[3] Ionagar no. 2, Consolidated Laboratories, Inc., Chicago Heights, Illinois.

TABLE 1

Feeding schedule and dietary sequences

Age of pigs	Vol. fed/day	Composition		
		Egg[1]	Cow's colostrum (C C)	Cow's milk
days	*ml*			
1	300	50 ml egg + 50 ml milk[2]	100 ml CC	100 ml milk + 7 gm MS[3]
2	400	50 ml egg + 50 ml milk	100 ml CC	100 ml milk + 7 gm MS
3	400	25 ml egg + 75 ml milk	50 ml CC + 50 ml milk	100 ml milk + 3.5 gm MS
4	500	25 ml egg + 75 ml milk	50 ml CC + 50 ml milk	100 ml milk + 3.5 gm MS
5	600	10 ml egg + 90 ml milk	25 ml CC + 75 ml milk	100 ml milk + 1.7 gm MS
6	600	10 ml egg + 90 ml milk	25 ml CC + 75 ml milk	100 ml milk + 1.7 gm MS
7	600	100 ml milk	100 ml milk	100 ml milk
8	ad libitum	100 ml milk	100 ml milk	100 ml milk

[1] Fresh whole egg.
[2] Fresh cow's milk fortified with cod liver oil and 60 ppm of iron and 5 ppm of copper on moisture-free basis.
[3] MS = nonfat dried milk solids.

for 4 hours in distilled water before drying and staining). The PVP, migrating as a very sharp band with mobility similar to γ-globulin, was visualized easily since piglets fed PVP were colostrum-free, hence, γ-globulin-free.

Pigs. A total of 32 pigs from 6 sows were used in the experiments. Ten were fed cow's milk fortified with avian eggs; 8, cow's colostrum; 6, cow's milk fortified with milk solids; 4, cow's milk fortified with PVP; and 4, a diet consisting solely of avian eggs. All pigs were assigned randomly to the various diets. Feeding and handling of pigs was similar to that previously reported (Lecce and Matrone, '60). Animals were bled at zero, 2, 4, 8 and 14 days of age.

Diets. Initially, to increase the possibility of detecting absorbed egg proteins, piglets were fed undiluted avian eggs from the time of birth. The serum from these pigs was analyzed for the presence of egg proteins by immunoelectrophoresis. Since all pigs died within 5 days when fed this whole-egg diet, it was modified as indicated in table 1.

Skim milk solids were added to the cow's milk diet so that it would compare roughly with the total protein solids present in the egg and colostrum diet.

Cow's colostrum used in this study represents a pool of the first day secretions obtained from a minimum of 12 cows.

PVP was fed at a concentration of 8% in cow's milk diluted 1:2 with water.

Weight gain-viability. Pigs surviving the various diets did not vary appreciably in weight; thus, it was believed an expression including weight gain and piglet survival would more accurately mirror this area of performance (weight gain-viability = mean weight of pigs × percentage of survival).

RESULTS

Specificity of early absorption phenomenon as demonstrated by immunoelectrophoresis and agar electrophoresis. Preliminary evidence using a ring precipitation test showed egg proteins and bovine proteins in sera from pigs fed those proteins from time of birth. Immunoelectrophoresis was used to determine specifically which of the proteins present in egg and cow's colostrum were being absorbed and thereby contributing antigens to the precipitation.

Egg white has three distinct electrophoretic protein zones — ovalbumin, ovoglobulin, and conalbumin migrating as a fast γ-globulin (fig. 1B). Minor antigenically-distinct protein fractions occurred within these major fractions as evidenced by 6 distinct arcs of precipitation (top half of slide in fig. 1A). Similarly, cow's colostrum showed two distinct electrophoretic protein fractions (fig. 2B) and 5 antigenically distinct protein fractions (top half of fig. 2A).

Thus, it was felt that cow's colostrum and avian eggs, with their many different protein fractions, would lend themselves to testing whether gamma-like globulins were preferentially absorbed or whether the absorption was qualitatively nonselec-

tive. As can be seen from the immunoelectrophoretic analyses of serum obtained from neonatal pigs fed egg (bottom half of fig. 1A) and cow's colostrum (bottom half of fig. 2A), the absorption process seemed nonselective. In both cases, γ-globulin and conalbumin were detected; however, so were fractions corresponding to lactalbumin and ovalbumin. The nonselective nature of absorption was emphasized further by data showing absorbed PVP in the sera of piglets fed this blood plasma extender (fig. 3).

Effect of feeding nonporcine proteins on the maturation of the piglet's serum protein profile. An examination of serum profiles of two-day-old pigs fed diets containing bovine proteins and avian proteins revealed that these foreign proteins affected the profile initially in a manner similar to sow's colostrum. In the case of the cow's colostrum (fig. 4A), as com-

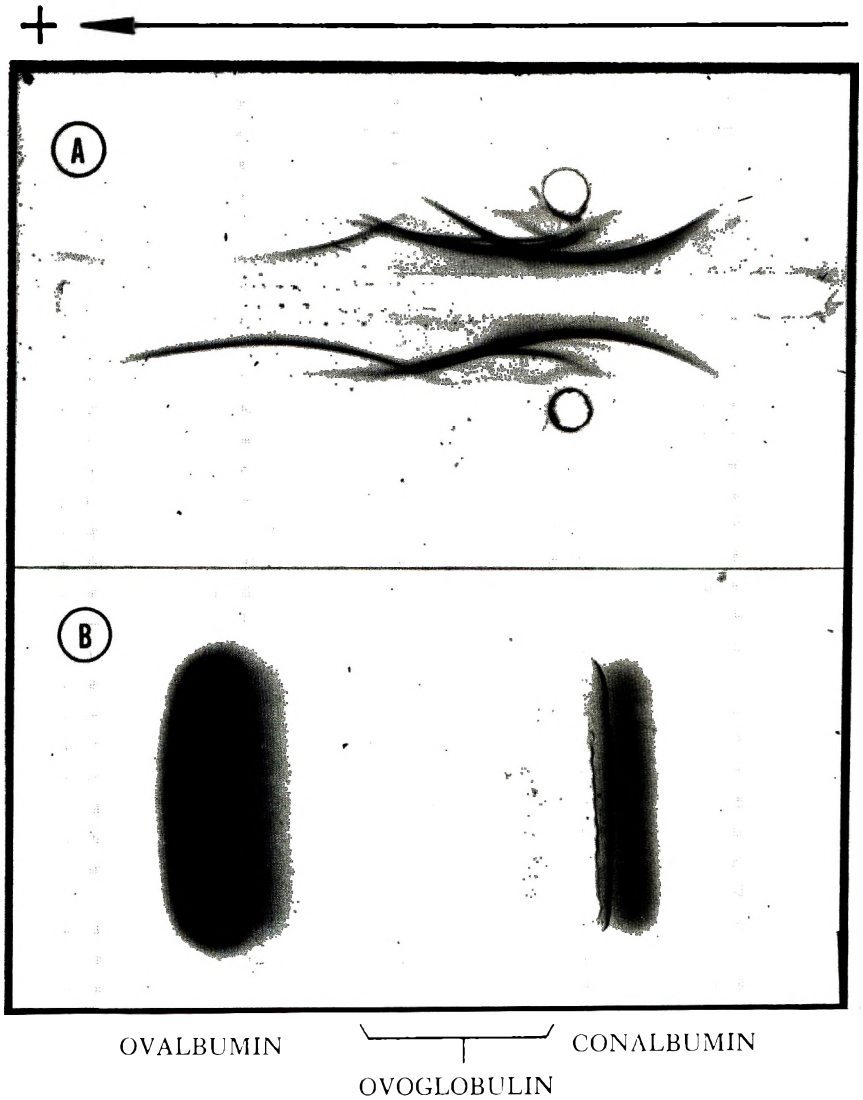

Fig. 1 A, Immunoelectrophoresis for the detection of egg proteins. Top hole, egg white; bottom hole, serum from pig fed eggs; center trough, anti-egg white serum. B, Agar electrophoresis of egg white proteins. Arrow indicates direction of electrophoretic mobility.

pared with the milk controls (fig. 4C), increases were found in the β-, γ-regions, as well as increases in TCA precipitable protein and total protein. Like changes were marked in pigs fed the whole-egg diet (fig. 5) and less intense in pigs fed whole egg diluted with cow's milk (fig. 4B).

With respect to the effect of nonporcine proteins on the maturation process, pigs weaned at birth to cow's colostrum (fig. 4A) did not exhibit the delay seen in pigs weaned immediately to milk (fig. 4C). That is, not only did it appear at two days that piglets had absorbed cow's colostral proteins, but also from two days on, an uninterrupted development toward a mature profile occurred. These increases in albumin, total proteins, and protein precipitated by TCA and decreases in the α-globulin were the same kinds of serum protein changes seen in pigs nursing the sow (Lecce and Matrone, '60). Pigs nurs-

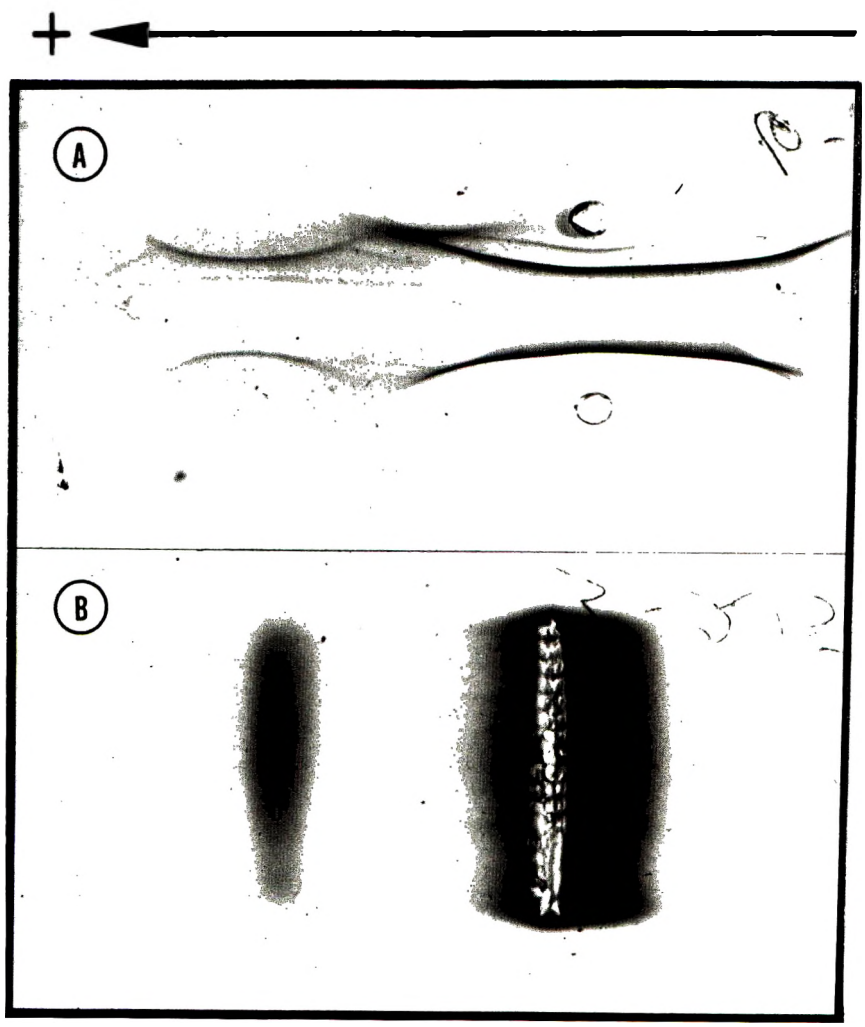

Fig. 2 A, Immunoelectrophoresis for the detection of bovine colostral proteins. Top hole, bovine colostrum; bottom hole, serum from pig fed bovine colostrum; center trough, anti-bovine whey serum. B, Agar electrophoresis of bovine colostral whey. Arrow indicates direction of electrophoretic mobility.

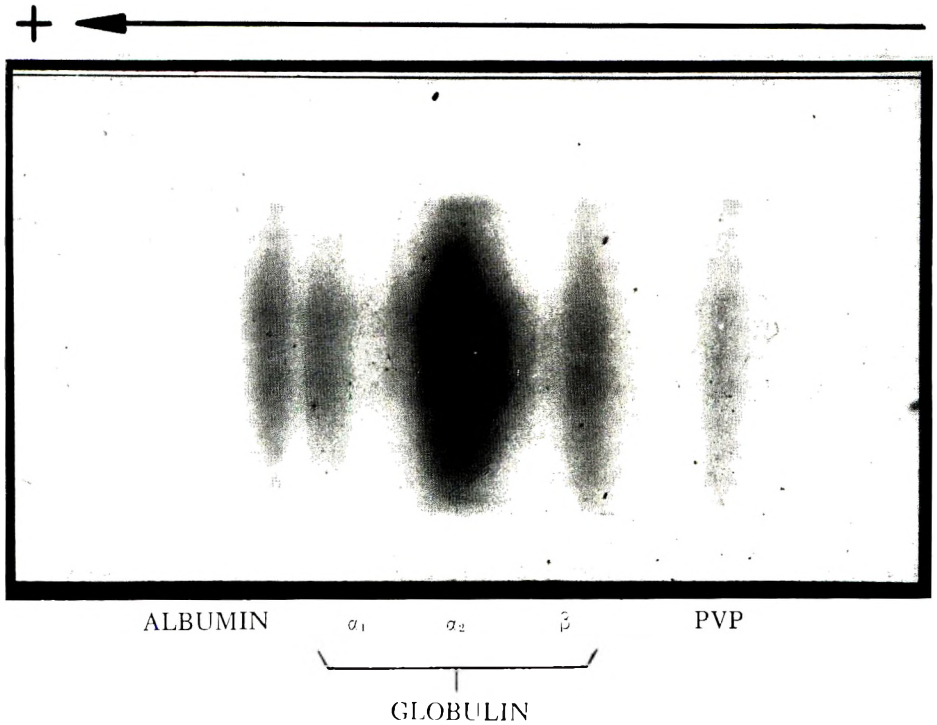

Fig. 3 Agar electrophoresis of serum from two-day-old piglet fed synthetic plasma extender (PVP). Arrow indicates direction of electrophoretic mobility.

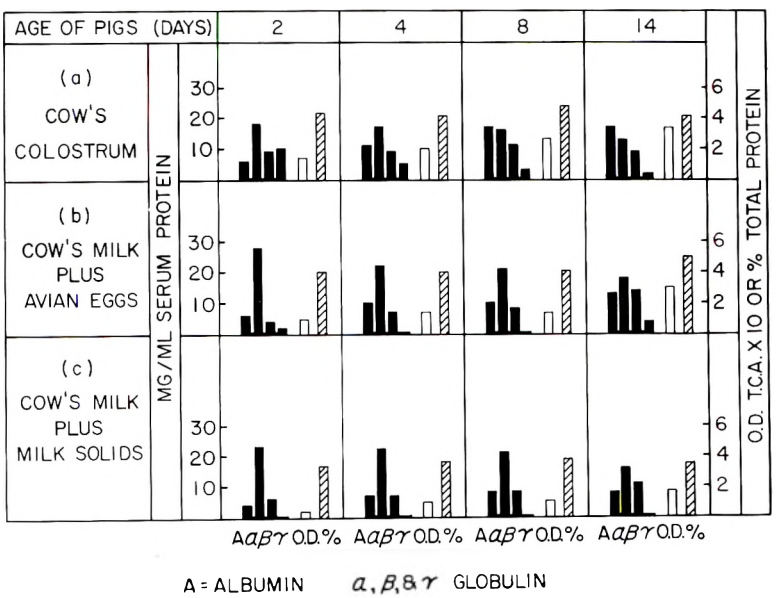

Fig. 4 The effect on the maturation of the serum protein profile of feeding neonatal pigs cow's colostrum, avian eggs in cow's milk, and cow's milk plus milk solids. OD indicates optical density of trichloroacetic acid (TCA) precipitate; % indicates percentage of protein.

ing the sow, however, sustained these changes at a higher level.

Although the data indicated egg proteins also were absorbed within the first two days of the piglet's life, there was no indication that they had any favorable influence on serum protein maturation process (fig. 4B). There was a latency of development as was evidenced by the immature profile at 8 days, namely, low albumin, β-globulin, total serum proteins, TCA precipitable proteins, and high α-globulin. There was virtually no difference in the results with pigs fed the egg diet and with the control pigs fed cow's milk (fig. 4C).

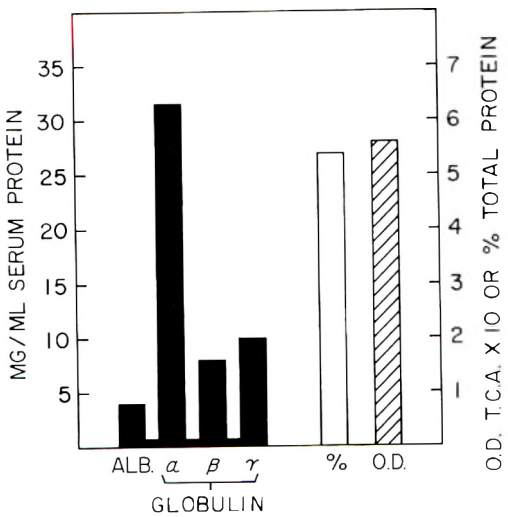

Fig. 5 Serum protein profile of two-day-old pigs fed avian eggs solely. OD indicates optical density of trichloroacetic acid (TCA) precipitate; % indicates percentage of protein.

Weight gain-viability. Weight gain-viability and maturation of serum proteins were closely paralleled. Those pigs (colostrum-fed) able to continue the maturation process after the initial absorption of proteins gained the most with an average weight of 1.02 kg at birth and 3.3 kg at 14 days. None of this group died. On the other hand, pigs fed cow's milk fortified with egg also had favorable initial changes but were unable to continue the maturation without delay. Pigs in this group weighed 1.07 kg at birth and 2.51 kg at 14 days. They did not gain as much as those fed colostrum, and 60% were dead by two weeks. In this respect, pigs fed egg performed no better than pigs weaned immediately to cow's milk, since 57% of the pigs fed milk died. Pigs in this

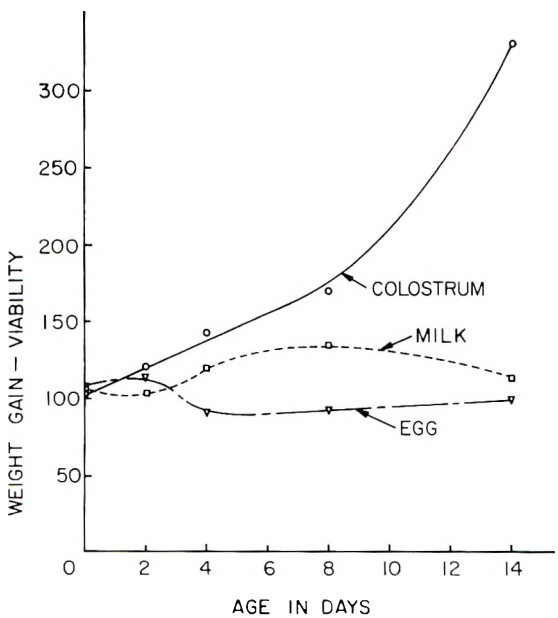

Fig. 6 Weight gain-viability of piglets fed cow's colostrum, avian eggs in cow's milk, and cow's milk plus milk solids.

group, averaging 1.06 kg at birth, weighed 2.62 kg at 14 days. These data expressed as weight gain-viability clearly illustrated the difference in performance between the various diets (fig. 6).

DISCUSSION

For reasons stated in the introduction, electrophoresis does not readily lend itself to the detection in piglets of absorbed proteins other than in the β-γ-globulin zone. This was confirmed herein with respect to the results obtained with pigs fed egg proteins and cow's colostrum. The electrophoretic patterns clearly showed, in egg-fed pigs, absorbed conalbumin (mobility similar to fast γ-globulin) and, in cow's-colostrum-fed pigs, absorbed γ-globulin. However, the other protein fractions present in egg and colostrum were not clearly detected. With these data one is faced with the choice that either gamma-like globulins are preferentially absorbed, or electrophoresis is too crude to detect minor changes in pre-existing serum protein electrophoretic fractions.

Immunoelectrophoresis, combining the resolving power of electrophoresis with the sensitivity and specificity of serology, demonstrated further the absorption of unaltered proteins from the gut of the piglet and clearly indicated the nonspecific nature of the absorption phenomenon. This was true with regard to the kinds of proteins absorbed (albumin and globulins) and the genetic source of protein (bovine colostral proteins, avian egg proteins and porcine proteins). Major and minor amounts of antigenically-distinct proteins fed the piglet were demonstrable subsequently in the piglet's serum. The nonspecific nature of this absorption was emphasized further by the fact that PVP (a nonprotein, high-molecular-weight blood plasma extender) was absorbed also. This lack of specificity is not peculiar to the piglet, since similar results were obtained with calves fed avian eggs[1] and dextran (Balfour and Comline, '60).

The nonspecific nature of the absorption phenomenon makes it easy, by feeding egg proteins, to mimic the initial changes seen in piglets nursing the sow. These pigs fed egg in cow's milk, however, were unable to sustain these initial changes, nor were they able to continue the maturation process without delay. Weight gain and death losses paralleled the poor weight gain and high death losses seen in control pigs weaned immediately to cow's milk. In contrast, littermates handled in a like manner, except fed initially cow's colostrum and subsequently weaned to cow's milk had immediate and continuing changes in serum protein profiles as well as favorable weight gains and viability.

These results indicate that cow's colostral proteins are enough like sow's colostral proteins that they are physiologically useful once absorbed, whereas egg proteins are not. In addition, cow's colostrum along with sow's colostrum not only promotes the initial changes occurring in the first phase of maturation but also contains a factor(s) or a balance of nutrients that influences the second phase or the continuing changes toward the development of a mature serum protein profile (Lecce and Matrone, '61). This similarity to sow's colostrum markedly enhances the experimental value of cow's colostrum.

SUMMARY

It has been demonstrated with agar and immunoelectrophoresis that the protein absorption mechanism operating within the first 36 hours of a piglet's life is qualitatively nonselective. Proteins from such phylogenentically different sources as chickens and cows, as well as different kinds of proteins (albumins and globulins), were absorbed by the neonatal pig. The nonselective nature of this absorption mechanism was emphasized further by the fact that a synthetic, high-molecular-weight, blood plasma extender (polyvinylpyrrolidone) also was absorbed by the piglet during this initial phase.

Piglets fed cow's colostrum had an uninterrupted maturation of the serum protein profile, superior weight gain and viability. Pigs fed avian eggs in cow's milk initially had similar serum protein changes, resulting from the absorption of egg proteins. This was followed by a delayed maturation of the serum protein profile, inferior weight gain, and poor viability. In this respect, the pigs fed egg per-

[1] Unpublished data.

formed as poorly as the control piglets fed cow's milk.

ACKNOWLEDGMENT

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Porcine Neonatal Nutrition: Effect of Weaning Time on the Maturation of the Serum Protein Profile[1]

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In a previous publication the point was stressed that pigs are born deficient in blood serum proteins (Lecce and Matrone, '60). Mainly, piglet's serum lacks γ-globulin and is extremely low in albumin and β-globulin. The major serum proteins, accounting for 70 to 80%, migrate in the α-globulin zone and are not precipitated by trichloroacetic acid. In addition, piglet's serum contains less than half the adult's total serum protein. This serum protein profile of birth was termed "immature" and the adult profile, "mature." The sequential changes involved in developing from the immature to the mature profile found in piglets were reminiscent of those reported to occur in fetal goats and sheep (Barboriak et al., '58a, '58b; Meschia, '55).

Nursing pigs had immediate changes in blood serum proteins, and in less than two weeks the serum profile was similar to an adult profile. The "maturation process" was delayed approximately a week in the pigs fed cow's milk, two weeks in those fed an artificial milk, and no maturation was seen in pigs eating an "amino acid milk" (Lecce and Matrone, '60).

The sow, through her mammary secretions, seemed to supply a factor(s) that promoted rapid, sequential changes in the piglet's serum proteins; and the piglet had a latency in the development of a mature serum profile unless he had access to this factor(s). The purpose of the experiments reported herein was to gain insight into the means whereby the sow influences the maturation of the serum protein profile. To pursue this goal, it was decided to define the amount of nursing (degree of exposure to sow's maturation factor(s)), required to overcome the latency in the piglet's protein metabolism. This was done by determining the extent of the serum protein changes occurring before the nursing piglet could be weaned from sow's milk to other food, without delaying the maturation process.

EXPERIMENTAL

Unless indicated to the contrary in the figures, pigs (with the exception of those weaned at 14 days) were bled at birth and at one, 4, 8, 14, and 21 days of age. Those pigs weaned at 14 days were bled approximately every 4 days until the experiment terminated at 52 days. Blood was analyzed for serum proteins, using paper electrophoretic techniques. The total serum proteins and the amount of the proteins precipitated by trichloroacetic acid (TCA) also were determined. Thus, three measurements characterized the serum protein profile: (1) milligrams per milliliter of albumin, α-, β-, and γ-globulin; (2) percentage of total protein; and (3) optical density (OD) of TCA precipitate. Details of the blood analyses, as well as the diets and methods of securing, handling and feeding the piglets, have been published previously (Lecce and Matrone, '60).

A total of 22 pigs from 5 litters was used in this study. Each pig was assigned randomly to the following treatments:

1. Eight pigs nursed the sow for the first 24 hours only; then 4 were switched to the amino acid diet[2] and 4 to the fortified cow's milk diet.

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[1] Contribution from the Animal Industry Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as paper no. 1221 of the Journal Series.

[2] Enzymatic casein and lactalbumin hydrolyzate, Nutritional Biochemicals Corporation, Cleveland.

2. Four pigs were allowed to nurse their sow for the first 4 days before weaning two pigs to the amino acid diet and two to the fortified cow's milk diet.

3. Six pigs nursed their sow for the first 8 days before changing two pigs to the amino acid diet and 4 pigs to the fortified cow's milk diet.

4. Four pigs nursed their sow for the first 14 days before weaning two pigs to the amino acid diet and two pigs to cow's milk diet.

RESULTS

As noted before (Lecce and Matrone, '60), pigs nursing the sow had rather intense, immediate, and continuous changes

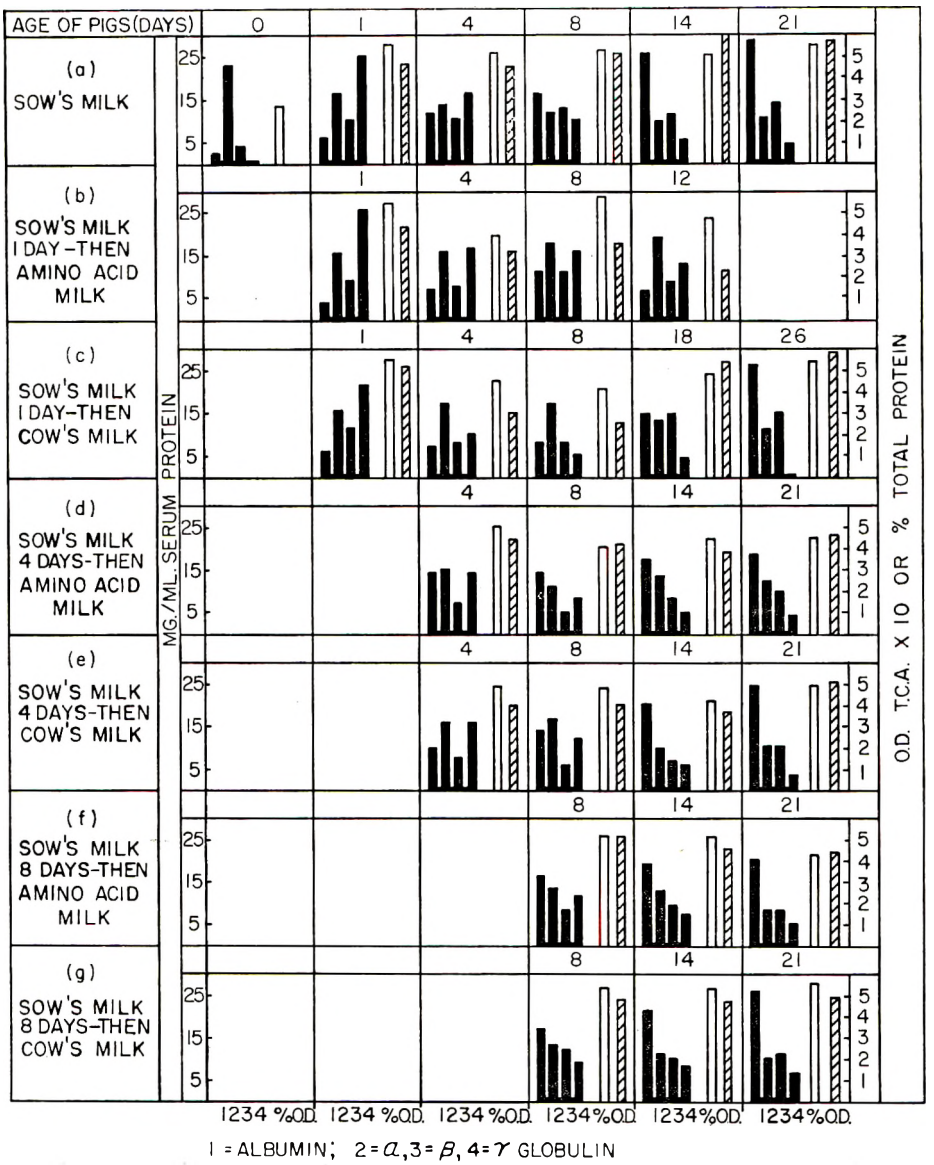

Fig. 1 The effect on the maturation of the serum protein profile of weaning piglets at one, 4 and 8 days to an amino acid diet and a cow's milk diet. OD indicates optical density of trichloroacetic acid precipitate; % indicates percentage of protein.

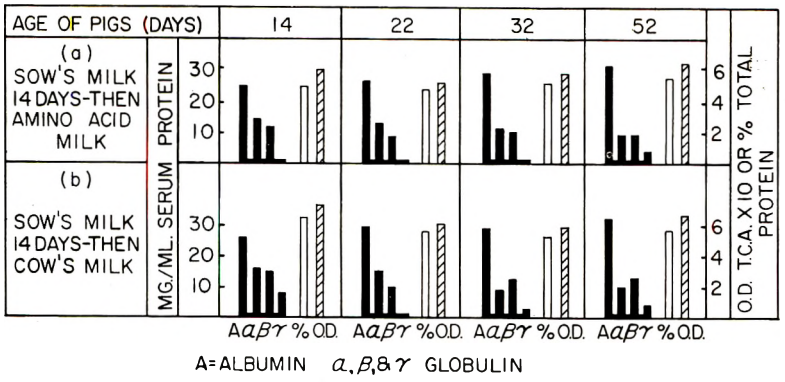

Fig. 2 Serum protein profiles of pigs weaned at two weeks to an amino acid diet and a cow's milk diet. OD indicates optical density of trichloroacetic acid precipitate; % indicates percentage of protein.

in serum proteins (fig. 1a). By 14 days the piglets had serum profiles similar to that of an adult.

Pigs allowed to nurse the sow for the first 24 hours (fig. 1b) and then switched to the amino acid milk did not hold the advantages of the immediate changes occurring from this one day of nursing, since the maturation process seemed to stop and even regressed. Half of the pigs that were changed over to the amino acid diet died by the 7th day, and all were dead by the 12th day. With the pigs switched to cow's milk (fig. 1c), only one out of 4 died (7th day). The serum protein maturation process also seemed to stop and then exhibit a latent period similar to that seen in pigs fed solely on the cow's milk from the time of birth (Lecce and Matrone, '60). The delay in the maturation of the serum protein profile in those pigs weaned to cow's milk at 24 hours was evidenced by a stabilized low ratio of albumin to α-globulin occurring in the first 8 days (fig. 1c) and by the similarity of the 18-day serum profile with the 8-day serum profile in pigs continuously nursing the sow (fig. 1a).

In pigs allowed to nurse the sow for 4 days, this delay in the development of serum proteins was not evident. Both the pigs weaned to amino acid milk (fig. 1d) and cow's milk (fig. 1e) maintained the changes that occurred while nursing the sow for the first 4 days and continued without apparent delay to develop a mature-looking serum protein profile. However, the albumin of the pigs switched to the amino acid diet seemed to have been stabilized at less than maximum level (19 mg/ml at 21 days). In contrast, pigs fed cow's milk did not have this limitation in amount of albumin (25 mg/ml at 21 days).

Much the same type of reaction as seen at 4 days occurred in the pigs nursing the sow for 8 days. The 8-day maturation changes were maintained, and serum protein development continued, but again the pigs weaned to the amino acid diet (fig. 1f) had less albumin (21 mg/ml at 21 days) than the pigs changed over to cow's milk (26 mg/ml at 21 days) (fig. 1g).

With those pigs nursing the sow for two weeks before weaning, their mature profile remained grossly unchanged for the 38-day testing period whether fed the amino

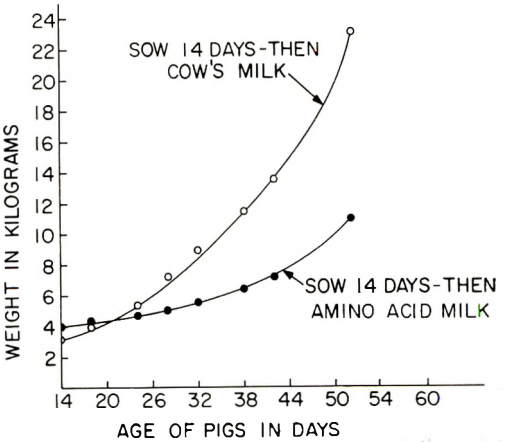

Fig. 3 Weight gains of pigs weaned at two weeks to an amino acid diet and a cow's milk diet.

acid diet (fig. 2a) or the cow's milk diet (fig. 2b). The pigs fed the amino acid diet, however, had diarrhea continuously and gained about half as much weight as their littermates fed milk (fig. 3).

DISCUSSION

From the data in this study and a previous study (Lecce and Matrone, '60), it appears that the proteins in sow's colostrum and milk are the source for the immediate changes observed in the maturing piglet's serum protein profile. These changes, no doubt, come from the piglet's ability to absorb, unaltered, proteins from the colostrum in the first 36 hours of its life (Lecce et al., '61). Having accounted for these immediate changes, however, the data herein indicate that beyond these initial changes, caused by absorption of whole proteins, there is still something unique about the sow's lacteal secretion that continues to influence changes toward a mature serum protein profile. This is inferred from the results obtained from the pigs that nursed the sow for one day and were switched to cow's milk and amino acid milk. Even though immediate changes occurred in the serum protein profile of these pigs, they were neither able to maintain these changes nor continue the serum protein maturation process without delay. Moreover, the amino acid diet did not support the life of the piglets in this nursing group.

After having nursed the sow for 4 days, however, the piglet apparently had developed sufficiently so that when weaned to cow's milk, maturation of the serum protein profile continued uninterrupted. Littermates weaned to the amino acid milk at 4 days seemed unable to utilize this food for further maturation as well as the pigs fed cow's milk (judging from the slower increase in serum albumin in the amino acid group). However, the amino acid diet did support the piglet's life at this stage of his physiologic maturity. Similar results were obtained for the piglets weaned at 8 days to cow's milk and amino acid milk. These results, plus the fact that pigs survived for at least 38 days without apparent changes in the mature serum protein profile when fed the amino acid diet, indicate that once a certain stage of maturity is reached, the amino acid diet is adequate for supporting life and maintaining the existing serum protein profile.

It might be concluded from these and other data (Lecce and Matrone, '60; Lecce et al., '61) that the sow influences the maturation of the piglet's serum protein profile in two ways, the first of which is by furnishing the proper physiologically active proteins that are absorbed unaltered by the piglet. This would constitute the first phase of the maturation. Secondly, the sow uniquely functions in maturation by supplying a factor(s) or balanced nutrients that provide for continuing changes in the piglet's serum proteins. This might be termed the second phase of maturation. The means whereby the sow functions in this second phase are not as clearly visualized as the means in the first phase are. However, a clue might be gleaned by contrasting the data obtained from the pigs weaned at one, 4 and 8 days (representing various stages in a maturing serum profile) to the amino acid milk with those weaned to the cow's milk. The less satisfactory results seen in piglets fed the amino acid milk imply that the protein needs of the piglet involve more than a supply of "amino acids." The possibility is raised that specific kinds of proteins (particularly rich in sow's milk) supply key peptide moieties required for protein synthesis. Future work will be designed to test this possibility.

SUMMARY

Pigs were weaned at one, 4, 8 and 14 days to fortified cow's milk and to an "amino acid" milk. Those weaned to cow's milk at one day experienced a delay in the maturation of their serum protein profile, even though marked changes toward a mature serum profile already had begun. Their response in serum protein development resembled the latent response seen in pigs weaned at the time of birth to cow's milk. Pigs weaned at one day to the amino acid milk showed an arrested

immature serum protein profile and eventually died.

Pigs weaned to cow's milk after 4 days of nursing were sufficiently mature physiologically so that no delay in serum protein development was observed. Pigs weaned at 4 days to the amino acid milk also developed a mature serum profile, although albumin did not reach as high a level as in the pigs fed cow's milk.

Results similar to those obtained in the pigs weaned at 4 days were seen in the pigs weaned at 8 days to cow's milk and amino acid milk.

Pigs, with mature serum protein profiles, weaned at 14 days maintained these profiles whether fed cow's milk or amino acid milk.

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Efficiency of Tryptophan as a Niacin Precursor in Man[1]

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The amino acid, tryptophan, has been shown to be a precursor of the vitamin, niacin, in man and in many animal species (Sarett and Goldsmith, '47, '49; Perlzweig et al., '47). Krehl and associates ('50) studied the efficiency of tryptophan as a niacin precursor in the rat. They found that a dose of 10 mg of tryptophan was comparable to 0.2 mg of niacin for the correction of niacin deficiency under the dietary conditions of their experiments, i.e., the efficiency of conversion was of the order of 50 to 1. Horwitt et al. ('55, '56), in an investigation of niacin-tryptophan relationships in a group of male subjects at Elgin State Hospital, found a conversion ratio of tryptophan to niacin of about 60 to 1. The efficiency of conversion of tryptophan to niacin in man was investigated in our laboratory as part of an extensive study of human niacin requirement which was initiated some years ago (Goldsmith et al., '52). A preliminary report of our observations[2] indicated that approximately 55 mg of tryptophan was the equivalent of 1 mg of niacin. The present paper reports the results which were obtained in 19 experiments with 14 adult female subjects.

METHODS

All subjects were maintained in a metabolism ward with controlled diets of low or moderate niacin and tryptophan content. Diets low in these nutrients furnished approximately 5.0 mg of niacin and 200 mg of tryptophan daily; those containing moderate amounts furnished 10 mg of niacin and 1000 mg of tryptophan. Urinary excretion of niacin and tryptophan metabolites was determined during (1) a control period using the diet alone, and (2) one or more experimental periods in which the diet was supplemented with 10 to 30 mg of niacinamide daily and (3) one or more experimental periods in which the diet was supplemented with 2 to 6 gm of DL-tryptophan daily. One experiment was conducted with each of the following supplements: 75 mg of niacinamide, 150 mg of niacinamide and 1 gm of L-tryptophan. The control periods and those in which supplements were administered were of two to three weeks' duration in most instances. Metabolites of niacin which were measured in the urine included niacin, N^1-methylnicotinamide (N^1–Me), and the pyridone of N^1-Me (pyridone). Excretion of quinolinic acid, total tryptophan and an ether extractable, tryptophan-like compound also were determined. Methods used for these determinations were those which have been described previously (Goldsmith et al., '52). The efficiency of conversion of tryptophan to niacin was calculated by comparison of excretion of niacin metabolites after administration of niacinamide with excretion after administration of tryptophan.

RESULTS

Nineteen experiments were carried out with 14 subjects. Nine subjects were studied during supplementation with niacinamide at one level and with tryptophan at one level. One subject (no. 4) received supplements of tryptophan at two levels and of niacinamide at one level, and 4 subjects (nos. 6, 9, 10, 13) received supplements of niacinamide at two levels and of trypto-

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[1] This study was supported by grants from the Nutrition Foundation, the Williams Waterman Fund of the Research Corporation and the National Institutes of Health, U. S. Public Health Service (grant A-1).

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

TABLE 1

Comparative data showing the amount of tryptophan equivalent to one milligram of niacin

Subjects no.	Niacinamide administered	Niacin excreted as metabolites	DL-Tryptophan administered	Tryptophan excreted as niacin metabolites	Tryptophan converted to niacin	Tryptophan equivalent to 1 mg niacin
	mg	%	*mg*	%	%	*mg*
			Normal group			
2	10	58.4	2000	2.2	3.8	44.5
5	10	88.2	2000	1.8	2.0	84.4
7	30	51.6	1000[1]	1.8	3.5	47.5
12	10	56.1	2000	1.4	2.5	68.4
13	10	40.6	2000	1.8	4.9	34.4
13	20	87.8	—	2.0	2.0	83.7
						Av. 60.5
			Rehabilitated group			
4[2]	30	59.6	3000	1.5	2.5	66.6
4[2]	—	—	6000	1.8	3.1	56.0
6	10	58.0	2000	1.7	2.9	57.7
6	20	54.3	—	—	3.1	54.1
8[2]	75	63.7	3000	2.1	3.3	51.3
9[2]	30	34.5	3000	1.7	5.0	33.7
9[2]	150	47.5	—	—	3.5	63.4
10	10	29.3	2000	1.2	4.1	40.7
10	20	42.5	—	—	2.8	59.0
14	10	53.8	2000	1.0	1.9	86.3
						Av. 56.9
			Diabetic group			
1	10	103.3	2000	3.5	3.5	48.3
3	10	22.6	2000	0.9	3.9	43.0
11	10	72.0	2000	3.3	4.6	36.3
						Av. 42.5

[1] L-Tryptophan administered.

[2] These subjects received a diet low in niacin and tryptophan and the remainder of the subjects received a diet which furnished moderate amounts of these nutrients.

phan at one level (table 1). Ten experiments involved 6 subjects who had been treated for pellagra. Determinations were made after rehabilitation was essentially complete. One experiment was conducted in each of three subjects who had diabetes mellitus. The disease was well controlled with diet alone in one instance and with diet and insulin in the other two. Six experiments were carried out in 5 subjects who had no evidence of nutritional or metabolic disease. The diets were low in niacin and tryptophan in 5 experiments and moderate in the other 14. Findings were similar with the two dietary regimens and among the several groups of subjects with the possible exception of those with diabetes mellitus.

Data which were obtained in the 19 experiments are given in table 1. The percentage of supplementary niacinamide which was excreted in the urine was calculated by subtracting the average excretion of niacin metabolites during the control period from the average excretion during the period of supplementation. Excretion during the first few days (usually two to 4) of each regimen was omitted from the calculation as it required this length of time for excretion to reach a relatively constant level. An average of 56.9% of the niacinamide which was administered was excreted as N^1-Me and pyridone with a wide range of values among the subjects studied. Pyridone excretion accounted for 70 to 90% of the increase in niacin metabolites in the urine following the supplements, N^1-Me excretion for 10 to 30%. The latter compound accounted for a larger percentage of the increased excretion at the higher dosage levels. In 4 subjects who received niacinamide at two levels of supplementation, there was a tendency for a larger percentage of the

TABLE 2

Percentage of dose of niacinamide excreted as metabolites in the urine of subjects who received two levels of supplementation

Subject no.	Niacinamide administered	Average excretion
	mg	%
6	10	58.0
	20	54.3
9	30	34.5
	150	47.5
10	10	29.3
	20	42.5
13	10	40.6
	20	87.8

dose to be excreted when the quantity administered was increased (table 2). When the group was considered as a whole, however, variation in percentage excretion could not be explained by the amount of niacinamide administered (table 3). Excretion is dependent in part on bodily stores of niacin and presumably also on metabolic characteristics of the individual, including his niacin requirement.

The percentage of supplementary tryptophan which was excreted in the urine as niacin metabolites varied from 0.9 to 3.5 with an average of 1.9 (table 1). Of this, an average of 1.5% (range 0.6 to 3.2) was present in the urine as pyridone and 0.4% (range 0.2 to 0.6) as N^1-Me. The percentage of tryptophan excreted in the urine as other derivatives was as follows: quinolinic acid, 0.1 to 1.1 (average 0.4), total tryptophan, 0.4 to 4.0 (average 2.7) and ether-extractable tryptophan, 0.4 to 3.1 (average 1.3).

The percentage of tryptophan which was converted to niacin was calculated on the assumption that the percentage of niacin excreted in the urine as metabolites would be the same when niacinamide *per se* was administered as when niacin was formed in the body from its precursor, tryptophan, both substances being given orally as dietary supplements. The increase in excretion of niacin metabolites (N^1-Me and pyridone) was calculated as micromoles of niacin in each instance. Free niacin was omitted from the calculations since no significant increases in excretion were observed after administration of tryptophan or niacinamide. In these calculations, it was assumed that D-tryptophan was not converted to niacin compounds. Findings determined in this manner are shown in table 4. The calculated extent of conversion of tryptophan to niacin was similar with tryptophan supplements ranging from 1 gm of L-tryptophan to 6 gm of DL-tryptophan. It should be noted that conversion was calculated by comparison with excretion of supplements of niacinamide ranging from 10 to 150 mg. The finding of essentially the same percentage conversion over this range of tryptophan supplementation, with varying amounts of niacinamide being used as a point of reference, lends support to the assumptions upon which the calculations were based.

The percentage of tryptophan which was converted to niacin in the 19 experiments varied from 1.9 to 5.0 with an average of 3.3 (table 1). The extent of conversion of tryptophan to niacin may be expressed in another way, namely, the milligrams of tryptophan which may be con-

TABLE 3

Percentage of various doses of niacinamide excreted in the urine as metabolites

No. of tests	Niacinamide administered	Average excretion of administered dose (expressed as niacin)		
		N^1-Me[1]	Pyridone	Total
	mg	%	%	%
10	10	5.3	53.0	58.3
3	20	12.5	49.0	61.5
3	30	10.2	38.3	48.5
1	75	8.8	54.9	63.7
1	150	11.2	36.3	47.5
	Average	7.8	49.1	56.9

[1] N^1-methylnicotinamide.

TABLE 4

Excretion of niacin metabolites after administration of DL-*tryptophan and of niacinamide*

No. of experiments	Tryptophan (T) and niacinamide (N) administered	Excretion of N^1-Me[1] + pyridone		Niacin formed from tryptophan (calculated in μmoles)	Tryptophan converted to niacin
		After tryptophan (μmoles expressed as niacin)	After niacinamide (μmoles expressed as niacin)		
					%
10	2 gm T 10 mg N	93.1	47.7	166.4	3.4
3	2 gm T 20 mg N	75.8	101.0	129.2	2.6
2	3 gm T 30 mg N	117.9	115.8	274.6	3.9
1	6 gm T 30 mg N	262.0	146.7	439.3	3.1
1	1 gm T[2] 30 mg N	88.8	126.7	172.0	3.5
1	3 gm T 150 mg N	125.7	583.8	265.0	3.5
1	3 gm T 75 mg N	152.8	392.6	239.0	3.3

[1] N^1-methylnicotinamide.
[2] L-Tryptophan.

sidered to be equivalent to 1 mg of niacin. This is shown also in table 1. An average of 55.8 mg of tryptophan was calculated to be equivalent to 1 mg of niacin with a range of 33.7 to 86.3 mg. The three patients with diabetes had values in the low normal range, 36 to 48 mg, with an average of 42.5 mg. The average conversion ratio in nondiabetic subjects was 58.7 mg. Further study will be necessary to determine whether this difference is significant.

DISCUSSION

The percentage of tryptophan converted to niacin varied considerably among the subjects studied. The same degree of variability was noted during the two dietary regimens, one of which furnished small amounts and the other, moderate amounts of niacin and tryptophan. Similar variability was observed with supplements of tryptophan which ranged from 1000 mg of L-tryptophan to 6000 mg of DL-tryptophan. In the one subject who was tested with two levels of tryptophan (3000 and 6000 mg), the conversion ratio was essentially the same during both experimental periods. Thus, the amount of tryptophan ingested appeared to have little influence on the conversion ratio under the conditions of these experiments. In three of 4 subjects who received two levels of niacin supplementation, the greater excretion of niacin metabolites with the larger supplement, accounts for the variation in the calculated conversion ratio in these subjects. Equally large variation, however, was found among other subjects irrespective of the amount of niacin which was administered. It seems likely that the extent of conversion of tryptophan to niacin may be a metabolic characteristic of the individual, at least when he is receiving an adequate supply of these nutrients as was the case in this study.

Findings in this investigation in women are similar to those obtained in men by Horwitt et al. ('56) who found a conversion ratio of tryptophan to niacin of the order of 60 to 1. These investigators also noted considerable variation among individuals. In Horwitt's studies, the basal diets furnished 5.8 mg of niacin and 265 mg of tryptophan. Some subjects received supplements of 100 mg of L-tryptophan or 10 mg of niacin. The conversion ratio was calculated on the basis of the increase in N^1-Me secretion resulting from the two supplements. Other subjects received 3 gm of albumin, which furnished about 200

mg of tryptophan, as a dietary supplement. Findings again indicated that approximately 60 mg of tryptophan was equivalent to 1 mg of niacin. The close agreement of our observations, using large supplements of tryptophan, with those of Horwitt and associates who administered small supplements, supports strongly the validity of a conversion ratio of approximately 60 to 1. This ratio should prove useful in calculating the niacin potential or "equivalent" of diets. Also, the results indicate that the efficiency of tryptophan as a niacin precursor in man is similar to that found in rats by Krehl et al. ('50).

SUMMARY

Nineteen experiments were conducted with 14 adult subjects to determine the efficiency of conversion of tryptophan to niacin in man. The conversion ratio was calculated by comparison of excretion of niacin metabolites (N^1-Me and pyridone) after administration of niacinamide with excretion after administration of tryptophan. The subjects were maintained with controlled diets furnishing low or moderate amounts of niacin and tryptophan. Calculations indicated that an average of 3.3% of the tryptophan which was administered was converted to niacin or, expressed in another way, that 55.8 mg of tryptophan was equivalent to 1 mg of niacin. The conversion ratio varied considerably among individual subjects: from 34 to 86 mg of tryptophan was found to be equivalent to 1 mg of niacin.

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The Response of Plasma Alkaline Phosphatase, Parathyroids and Blood and Bone Minerals to Calcium Intake in the Fowl[1]

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Changes in the dietary calcium level have been shown to have a profound influence on bone composition, parathyroid size and bone and plasma alkaline phosphatase. A reduction of bone ash and bone calcium in pullets upon calcium restriction was shown by Taylor and Moore ('56). Benoit and Clavert ('45) found that reduced dietary calcium levels would cause parathyroid enlargement in ducks. Plasma alkaline phosphatase was shown to be influenced by dietary calcium in the hen (Common, '36) and in sheep (Auchinachie and Emslie, '33). It was the purpose of the experiments described in this paper to investigate the interrelationships of the above mentioned parameters. Since it was shown with rabbits (Baumann and Sprinson, '39) that dietary phosphorus aggravated a calcium deficiency, the influence of dietary phosphorus in calcium restriction was also investigated.

EXPERIMENTAL AND RESULTS

Analytical procedures. Calcium was determined by an ethylenediaminetetraacetic acid (EDTA) titration using murexide and napthol green B as indicator (Hurwitz and Griminger, '60). For plasma calcium a direct titration was used. Plasma total phosphorus was determined after digestion of whole plasma with sulfuric acid and hydrogen peroxide. Plasma inorganic phosphorus was determined on the protein-free filtrate prepared by trichloroacetic acid (TCA) precipitation. Phosphorus analysis was carried out colorimetrically by the method of Gomori ('42). Plasma alkaline phosphatase was determined according to the method described in a technical bulletin,[2] using *p*-nitrophenol-phosphate as the substrate.

Tibias were cleaned of all adhering tissue, and dried overnight in an oven at 105°C. They were then weighed and ashed in a muffle furnace at approximately 800°C for three hours. The ash was weighed and dissolved in hydrochloric acid and aliquots were taken for phosphorus and calcium analyses.

Trial 1. The first experiment was undertaken in order to investigate calcium depletion in the laying hen as influenced by dietary phosphorus, with observations on the time sequence of changes in plasma alkaline phosphatase, parathyroid size and plasma inorganic phosphorus.

After several preliminary experiments had been carried out to observe individually the parameters under investigation, 20 White Leghorn pullets, 9 months old, were selected on the basis of satisfactory egg production. The pullets were divided randomly into 4 groups of 5 birds each and placed in individual laying cages situated in a constant environment room (70°F, and 14 hours of artificial illumination daily). Water and feed were provided ad libitum.

The composition of the diets[3] together with the analyses of their calcium and phosphorus contents is shown in table 1. One group of 5 pullets (lot 1) received the calcium-free, low-phosphorus diet (diet 1); one group (lot 3) was fed a positive control

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[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station.

[2] Sigma Chemical Company 1960 The colorimetric determination of phosphatase. Technical Bull. no. 104, rev. ed., St. Louis.

[3] The authors are indebted to Merck Sharp and Dohme, Rahway, New Jersey, and to Stabilized Vitamins, Inc., Garfield, New Jersey, for material used in this study.

diet (diet 3, containing adequate calcium and phosphorus), and two groups (lot 2) received the calcium-free diet supplemented with sources of inorganic phosphorus (diet 2).

During a preliminary three-day period all pullets were fed the positive control diet. Thereafter experimental diets were fed continuously until no eggs were produced by the calcium-depleted animals for two consecutive days. Egg production and egg weights were recorded for the preliminary and experimental period. Blood samples were obtained from the brachial vein of all hens at the end of the preliminary period and after the first day of feeding the experimental diets; later, blood was obtained from each group on alternate days

TABLE 1

Composition of experimental diets for trial 1[1]

Constant	All diets
	%
Soybean oil meal (50% protein)	20.00
Egg albumen, dried[2]	8.00
Corn oil, refined	3.00
Sodium chloride	0.30
Choline chloride	0.10
Vitamin A, D and E conc.[3]	0.10
Vitamin mix[3]	0.15

Variable	Diet 1	Diet 2	Diet 3
K_2HPO_4	—	1.10	1.10
$NaH_2PO_4 \cdot H_2O$	—	1.10	1.10
$CaCO_3$	—	—	6.50
Glucose monohydrate	68.35	66.15	59.65
Total	100.00	100.00	100.00

[1] Protein content of the diets (calculated), 16%. Calcium content (assayed): diet 1, 0.03%; diet 2, 0.03%; diet 3, 2.73%. Phosphorus content (assayed): diet 1, 0.12%; diet 2, 0.49%; diet 3, 0.50%.

[2] Egg white solids, Henningsen, Inc., New York.

[3] Fisher and Johnson ('56).

TABLE 2

Effect of calcium depletion on performance and plasma calcium and phosphorus of laying hens

Parameter	Time[1]	Dietary treatment[2]		
		1	2	3
Av. body weight, gm	Start period 2	1743 ± 244[3]	1753 ± 151	1710 ± 111
	End period 2	1650 ± 295	1654 ± 168	1747 ± 86
Av. feed consumption, gm/bird/day	Period 1	97	112	103
	Period 2	88	80	111
Av. egg production/ bird/period	Period 1	2.4 ± 0.5	2.4 ± 0.5	2.4 ± 0.5
	Period 2	4.0 ± 1.1	3.7 ± 0.9	5.2 ± 0.7
Plasma calcium mg/100 ml	Start period 2	21.4 ± 1.7	24.8 ± 2.8	21.0 ± 1.0
	End period 2	10.7 ± 3.4	12.8 ± 3.8	21.9 ± 1.8
Total plasma P mg/100 ml	Start period 2	39.6 ± 9.4	44.3 ± 7.6	33.5 ± 3.0
	End period 2	18.4 ± 6.8	21.5 ± 6.1	37.2 ± 8.6

[1] Period 1, preliminary period lasting three days; period 2, treatment period lasting 7 days.

[2] Lot 1, no added Ca or P (5 birds); lot 2, supplemental P only (10 birds); lot 3, supplemental Ca and P (5 birds).

[3] Mean ± standard deviation.

TABLE 3

Effect of calcium depletion on parathyroid weight and tibia components of laying hens

	Dietary treament[1]		
	1	2	3
	Parathyroids		
Fresh weight, mg	26.6 ± 6.7[2]	32.2 ± 10.2	10.9 ± 2.2
	Tibia		
Dry weight, gm	5.059 ± 1.261	5.029 ± 0.714	5.867 ± 0.508
Ash, gm	2.263 ± 0.536	2.283 ± 0.215	2.804 ± 0.359
Ash, % of dry weight	44.99 ± 4.21	45.77 ± 4.06	47.66 ± 3.57
P, mg	356 ± 79	359 ± 32	436 ± 59
P, % of dry weight	7.08 ± 0.65	7.21 ± 0.79	7.41 ± 0.52
Ca, mg	871 ± 199	882 ± 78	1083 ± 146
Ca, % of dry weight	17.34 ± 1.59	17.71 ± 1.81	18.41 ± 1.28
Ca:P ratio	2.45 ± 0.07	2.46 ± 0.06	2.48 ± 0.03

[1] See footnote 2, table 2. The depletion period lasted 7 days.
[2] Mean ± standard deviation.

in order to avoid possible adverse effects from excessive bleeding. Heparin was used as an anticoagulant.

At the end of the experimental period, all animals were killed with ether, the parathyroid glands were removed and weighed and the left tibias removed for calcium and phosphorus analyses.

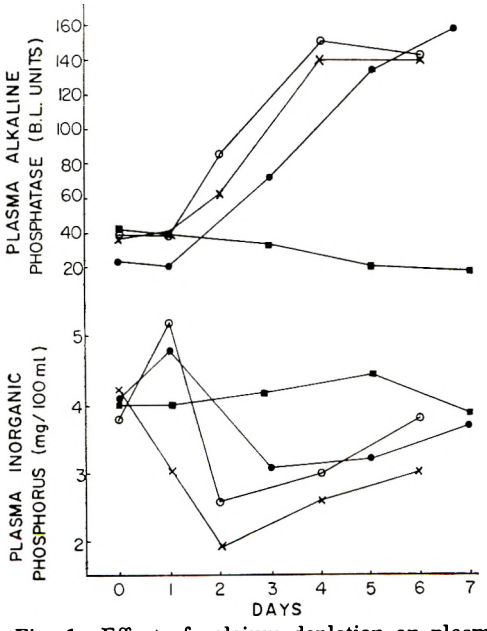

Fig. 1 Effect of calcium depletion on plasma alkaline phosphatase and plasma inorganic phosphorus. (×) denotes a group without supplemental calcium or phosphorus, (○) (●) groups receiving phosphorus only and (■) a group receiving both phosphorus and calcium (positive control).

For the presentation of body weight, feed consumption, egg production, parathyroid weights, and for tibia values, both groups of 5 birds receiving the diet deficient in calcium and supplemented with phosphorus were combined; whereas for the plasma inorganic phosphorus and alkaline phosphatase, the values are presented separately for each group.

Results of this experiment are presented in tables 2 and 3 and figure 1. During the control period little change in body weight occurred. During the experimental period the chickens in both calcium-depleted groups reduced their feed consumption and registered a moderate weight loss.

Egg production was similar in all groups during the control period. In the experimental period, the chickens in both low-calcium groups (with and without phosphorus) laid an average of 4 eggs before ceasing production. In the control group, egg production, as anticipated, continued undisturbed throughout the experimental period.

Plasma inorganic phosphorus values are shown in figure 1. While the control group showed little change, the group receiving neither calcium nor added phosphorus showed a decrease on the first day of treatment, reaching a minimum by the second day. After the second day, the inorganic phosphorus started to rise. In the groups receiving no calcium, but added phosphorus, there was an increase in the inorganic phosphorus level on the first day, followed by a decrease on the second day,

with a return to control values by the 6th and 7th day.

Plasma alkaline phosphatase values showed a slight tendency to decrease in the control group (fig. 1). In the deficient groups, the alkaline phosphatase level did not change during the first day, but in the succeeding three-day period increased markedly, reaching an apparent plateau by the 4th and 5th day.

Plasma calcium (table 2) did not change significantly in the control group, and decreased in all calcium-depleted groups to approximately half the original value, thus reaching the level of a nonlaying hen. This agrees with the observations of Deobald et al. ('38) and Urist ('59). Similar to plasma calcium, total plasma phosphorus decreased to about half of the original value. This may be due to a decline in lipophosphoprotein complex and phosphoprotein, which are major components of the total phosphorus in hen plasma. Since plasma inorganic phosphorus approximates only one tenth of the total phosphorus of the laying-hen's plasma, changes in the former would have little effect on total plasma phosphorus.

Parathyroid weight (table 3) was significantly ($P < 0.05$) greater in the calcium-depleted groups than in the control group. There was no significant difference, however, between the phosphorus-unsupplemented and the phosphorus-supplemented group. There was a significant difference between the control (lot 3) and both calcium-deficient groups, namely, with and without phosporus supplementation, in tibia weight, ash, calcium and phosphorus. No such difference was noted between the two phosphorus treatment groups ($P > 0.05$). When ash, calcium and phosphorus are expressed as a percentage of dry bone, the two calcium-deficient groups tended to show lower values (though not significantly so) as compared with the control group.

Trial 2. This experiment was designed to determine whether the response of plasma alkaline phosphatase to dietary calcium is an "all or none" effect, or whether a quantitative relationship exists between dietary calcium and plasma alkaline phosphatase in the laying hen. For this purpose 25 birds in good production were assigned to 5 groups on the basis of their plasma alkaline phosphatase levels so that the group averages for phosphatase were practically equal. The birds were managed as in the previous experiment and received diets with graded levels of calcium as shown in table 4. After 4 days the birds were bled from the brachial vein and plasma alkaline phosphatase was determined.

All hens except some of those getting the lowest calcium level laid three or more eggs during the 4-day experimental period. Alkaline phosphatase values, and a second degree curve fitted to these values, are shown in figure 2. With increasing levels of calcium, plasma alkaline phosphatase decreased reaching a minimum, by calculation, at the 2.60% level of dietary calcium.

Trial 3. This experiment was similar to the previous one, except that it was conducted with growing chicks. In addition to plasma alkaline phosphatase, growth and tibia ash, calcium and phosphorus were measured in order to correlate these parameters with changes in plasma alkaline phosphatase.

Male Vantress chicks were raised in growing batteries to one week of age with a practical growing mash. Fifty chicks were selected by discarding all chicks weighing below 95 and above 115 gm. The chicks were then assigned to 5 groups and received the diets shown in table 4. At two weeks of age all birds were bled by heart puncture and plasma alkaline phosphatase was determined. After feeding the birds the same diets for an additional week, they were weighed; 6 chicks from each lot were taken at random and their left tibia removed for chemical analysis. The results are presented in table 5 and figure 3. Body weight as well as tibia ash, calcium and phosphorus increased progressively with increased dietary calcium levels, whereas plasma alkaline phosphatase decreased. The equations of the curves of plasma alkaline phosphatase and the percentage of tibia calcium are presented in figure 3 and those for body

TABLE 4

Composition of experimental diets for trials 2 and 3

Constant	Trial 2	Trial 3
Soybean oil meal (50% protein)	30.00	45.00
Corn oil, refined	3.00	3.00
Choline chloride	0.10	0.10
Vitamin A, D and E conc.[1]	0.10	0.10
Vitamin mix[1]	0.15	0.15
DL-Methionine	0.10	0.25
Sodium chloride	0.30	—
Minerals[2]	—	0.50
KH_2PO_4	1.10	1.10
$NaH_2PO_4 \cdot H_2O$	1.10	1.10
Total	35.95	51.30

Variables	Lot				
	1	2	3	4	5
Trial 2					
$CaCO_3$	2.30	3.50	4.80	6.00	8.00
Glucose monohydrate	61.75	60.55	59.25	58.05	56.05
% Ca (assayed)	0.97	1.46	1.95	2.50	3.10
Trial 3					
$CaCO_3$	—	0.50	1.00	1.50	2.50
Glucose monohydrate	48.70	48.20	47.70	47.20	46.20
% Ca (calculated)	0.13	0.33	0.53	0.73	1.13

[1] Fisher and Johnson ('56).

[2] Supplied in gm/ kg diet: NaCl, 3; $Fe(C_6H_5O_7)_2$, 0.31; $MgSO_4$, 1.50; $MnSO_4$, 0.12; KI, 0.006; $CuSO_4$, 0.008; $ZnCO_3$, 0.06; and $Na_2MoO_4 \cdot 2H_2O$, 0.006.

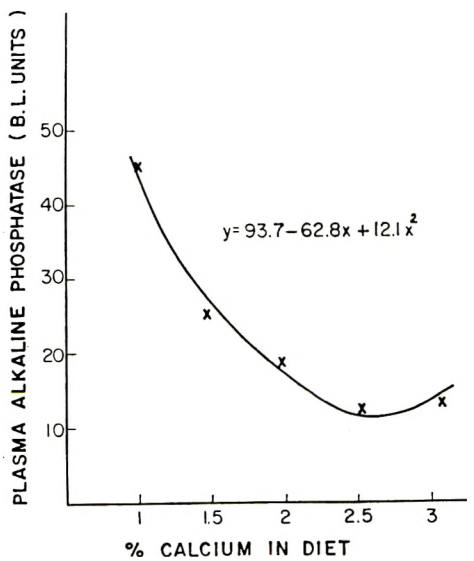

Fig. 2 Effect of graded levels of dietary calcium on plasma alkaline phosphatase of laying hens.

weight and percentage of tibia ash are as follows:

Body weight: $y = 202 + 271x - 129x^2$, with a maximum at 1.05% of dietary calcium.

Percentage of tibia ash: $y = 22.1 + 28.6x - 12.8x^2$, with a maximum at 1.12% of dietary calcium.

DISCUSSION

Robison ('23) suggested that alkaline phosphatase played an important role in the calcification of bone. In the chick, the plasma or serum alkaline phosphatase levels are directly related to the levels in bones (Motzok, '50a). The enzyme is elevated in cases of improper calcification such as in vitamin D deficiency (Motzok, '50a, b) or in diseases involving decalcification of bone such as osteoporosis (Kay, '32). Similarly, our findings indicate increased enzyme levels in plasma resulting from a dietary calcium deficiency.

TABLE 5

Effect of dietary calcium on growth, feed efficiency and bone composition in the growing chick[1]

% Dietary calcium	0.1	0.3	0.5	0.7	1.1
	mg	*mg*	*mg*	*mg*	*mg*
Tibia					
Dry weight	783 ± 62[2]	901 ± 192	1018 ± 139	1177 ± 156	1243 ± 134
Ash	199 ± 20	272 ± 58	344 ± 61	423 ± 56	473 ± 57
Calcium	63.6 ± 7.2	92.8 ± 21.7	123.5 ± 21.4	156.4 ± 19.0	171.1 ± 21.5
Phosphorus	30.7 ± 2.8	42.9 ± 10.3	53.5 ± 8.1	64.5 ± 8.0	725 ± 8.9
			Percentage of dry weight		
Ash	25.37 ± 0.86	30.22 ± 1.67	34.07 ± 2.39	35.94 ± 2.81	38.01 ± 1.17
Calcium	8.10 ± 0.36	10.28 ± 0.75	12.09 ± 0.77	13.30 ± 0.34	13.75 ± 0.42
Phosphorus	3.94 ± 0.04	4.75 ± 0.32	5.25 ± 0.28	5.48 ± 0.24	5.82 ± 0.18
Ca:P ratio	2.07 ± 0.06	2.16 ± 0.02	2.31 ± 0.08	2.39 ± 0.05	2.36 ± 0.02
Body weight, gm	238 ± 16	268 ± 46	318 ± 29	332 ± 25	324 ± 27
Feed efficiency	0.57	0.61	0.65	0.67	0.67

[1] Data are for three-week-old chicks (after being fed the experimental diets for two weeks), except for feed efficiency, which refers to the first week of the experimental diets only.

[2] Mean ± standard deviation.

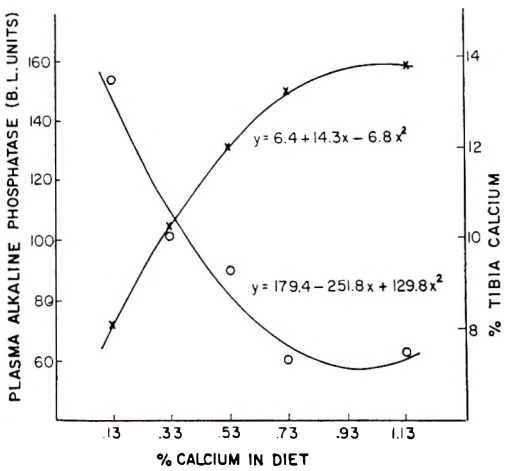

Fig. 3 Effect of graded levels of dietary calcium on plasma alkaline phosphatase and percentage of tibia calcium in the chick. (○) denotes plasma alkaline phosphatase and (×) denotes percentage of tibia calcium. Calculated minimum for alkaline phosphatase at 0.97% of dietary calcium, maximum for tibia calcium at 1.05% of dietary calcium.

There are marked alterations in plasma alkaline phosphatase levels of poultry associated with egg production. Plasma alkaline phosphatase levels increase when pullets commence laying (Auchinachie and Emslie, '34; Common, '36; Bell, '60). This increase was more pronounced when using a low-calcium diet. In the early stages of egg production, pullets are in negative calcium balance (Morgan et al., '42; Hurwitz and Griminger, '60) and draw upon their bone calcium reserves for shell formation. This negative balance is a possible explanation for the increased levels of plasma alkaline phosphatase observed upon commencement of egg production.

It is common knowledge that the breakdown of bone is associated with parathyroid activity (Neuman and Neuman, '58). Several authors (Williams and Watson, '41; Cantarow et al., '37; Ambroso et al., '58a,b) have reported that administration of parathyroid hormone resulted in elevated bone or plasma aklaline phosphatase levels. A similar observation was made by Gaillard ('60) with bone cultures. On the basis of these and our own findings it seems that plasma alkaline phosphatase, when reflecting the bone phosphatase level, is associated with decalcification, or insufficient calcification, conditions which both involve increased parathyroid activity. A quantitative relationship was shown to exist between the individual plasma alkaline phosphatase values and the corresponding wet weights of the parathyroid glands (fig. 4). The correlation coefficient between these two measurements was 0.728 (highly significant). Hence it is believed that plasma alkaline phosphatase, under experimental conditions in which changes in calcium metabolism are the variables, may serve as a criterion of parathyroid activity in the fowl. Moreover plasma alkaline phosphatase gives a graded response to dietary calcium, as shown with growing chicks (table 5 and fig. 3) and

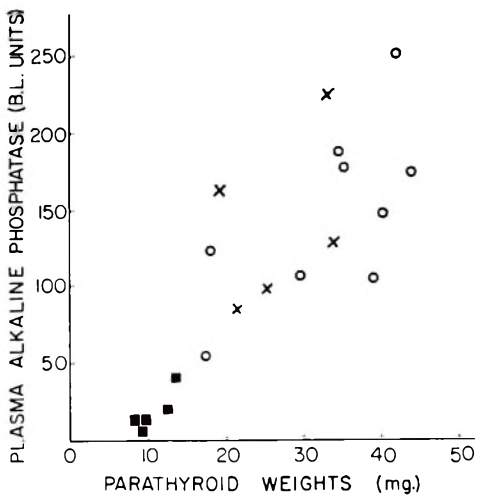

Fig. 4 Relationship between plasma alkaline phosphatase and wet weight of parathyroid glands. Symbols represent measurements on individual hens. Alkaline phosphatase values are those obtained just prior to killing of the bird. (×) denotes hens not receiving supplemental calcium or phosphorus, (○) hens receiving phosphorus only and (■) hens receiving both phosphorus and calcium (positive control).

laying hens (fig. 2). Thus, maximum calcification seemed to correspond in the chick with minimum plasma alkaline phosphatase, and the level of dietary calcium of 2.60% at which minimum alkaline phosphatase was indicated in the laying hen also falls within the range of what is believed to be the calcium requirement at this level of food intake. We therefore suggest that consideration should be given to the use of plasma alkaline phosphatase levels as a possible measure of calcium adequacy. The short duration of the required experiments would be a distinct advantage.

Plasma inorganic phosphorus appears to be influenced by the diet after one day of treatment, showing, as expected, a decrease in the group receiving neither added calcium nor phosphorus. In the group receiving phosphorus but no added calcium, the level increased since there was insufficient calcium available to permit deposition of phosphorus in bone. The fact that there was no apparent increase in plasma alkaline phosphatase after one day suggests that at that time no significant increase in parathyroid activity had taken place. After two days on the depletion regime, and coincident with a sharp increase in alkaline phosphatase there was a further decrease in inorganic phosphorus in the calcium-depleted group without phosphorus supplementation, and a sharp decrease in the calcium depleted group receiving phosphorus. This is believed to be due to the increased parathyroid activity which reflects the severe calcium deficiency resulting from the loss of appreciable amounts of calcium (approximately 7 gm per bird) for the formation of egg shells. No explanation can be offered at the present time for the apparent adaptation mechanism which increases plasma inorganic phosphorus levels again after the third day. Since a similar picture was observed in a study with adult males, we do not believe it to be related to the decline in egg production.

Tibias showed a significant loss of dry weight in the depleted hens. This loss in the course of the experiment (taken as the difference between the control and the calcium deficient animals) of approximately 800 mg can be accounted for in part by the loss of ash which was approximately 500 mg, indicating a 300-mg loss of organic matter. It is believed that this breakdown of bone is associated with parathyroid activity. It is possible, however, that not only parathyroid hormone but also the adrenal corticosteroids play a role in bone breakdown in birds, causing osteoporosis, as suggested by Urist ('59). In our experiment the loss of mineral matter is relatively greater than that of organic matrix, supporting the view of Neuman and Neuman ('58) that at least partial demineralization must precede the destruction of bone matrix. These results concerning the relative breakdown of bone mineral and matrix are consistent with those observed in other depletion experiments carried out previously in this laboratory.

Although similar results were obtained with growing chicks, the differences in bone weight and bone organic matter in these experiments may be attributed to differences in growth and body weight and not to total breakdown of bone. A major difference between the chicks and the hens lies in the response of the bone Ca:P ratio to the different calcium regimes.

While in the hens the Ca:P ratio was hardly altered (2.45 at the low calcium and 2.48 at the high calcium level), a graded response was obtained in the chick (from 2.01 at the lowest calcium level to 2.36 to 2.39 at the highest calcium level). This can be explained by the fact that in the hens, bone is actually broken down, whereas in the chick the build-up of the bone is impaired, resulting in a relatively greater deposition of phosphorus.

Inorganic phosphorus supplementation of the calcium-deficient diet had no effect on calcium depletion as expressed by any of the parameters measured; hence, the presence of phosphorus did not seem to aggravate calcium deficiency in the laying hen. This is in contrast with results obtained in experimentation with rabbits (Baumann and Sprinson, '39) where the addition of phosphorus to calcium-deficient diets seemed necessary for the production of hypertrophy of the parathyroids; there, phosphorus was considered a factor in the reduction of the absorption of the small amounts of calcium present in the gut. In the hen, however, calcium depletion through deposition in the egg shell is of such magnitude that this effect will be relatively unimportant.

SUMMARY

1. Three experiments, two with laying hens and one with growing chicks, were conducted to evaluate the relationships between plasma alkaline phosphatase and plasma inorganic phosphorus, bone constituents and parathyroid size.

2. Enlarged parathyroids, elevated plasma alkaline phosphatase and loss of bone material were found in calcium-deficient hens irrespective of supplementation with phosphorus, suggesting that supplemental phosphorus had no influence on calcium depletion in laying hens.

3. The experimental results indicate that decalcification rather than calcification of bone is associated with increased alkaline phosphatase levels.

4. In hens, a relationship was observed between the weights of the parathyroid glands of the individual birds and their plasma alkaline phosphatase levels, suggesting that plasma alkaline phosphatase, at least under our experimental conditions, is indicative of parathyroid activity.

5. Both in the laying hen and the growing chick, plasma alkaline phosphatase was found to decrease as dietary calcium increased, reaching a minimum in the range of the probable calcium requirement. The possible use of plasma alkaline phosphatase activity for the determination of calcium adequacy is proposed.

6. In hens, both bone ash and bone organic matter appeared to decrease with calcium depletion. Little change was observed in the bone Ca:P ratio in these birds; in chicks, however, a smaller ratio was obtained at low levels of dietary calcium.

ACKNOWLEDGMENT

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Amino Acid Requirements of Children: Isoleucine and Leucine

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The present paper is a continuation of the studies in this laboratory on the amino acid requirements of children and is concerned with the minimal needs for isoleucine and leucine.

EXPERIMENTAL PROCEDURE AND RESULTS

Experiment 1. The methods employed were described in detail in a previous report (Nakagawa et al., '60); therefore, they are discussed here only briefly. The basal diet consisted of cornstarch, sugar and butterfat, and a mineral and vitamin mixture. The protein was supplied by mixtures of highly purified amino acids (table 1).

Nonessential amino acids were administered in the form of pills, and the essential amino acid mixtures were swallowed by the use of wafers. Total daily intakes of amino acids were divided into three portions and given with other dietary constituents for meals. Three 11-year-old boys served as subjects, living in an institute under our supervision. A three-day period was allowed for the children to become accustomed to the test diets and also to living in a laboratory, preceding the collection of excreta. Nitrogen of diet, urine and feces was analyzed by the macroKjeldahl method, and the nitrogen balance was determined. Excretion of creatine and creatinine was also measured. Although all analyses were made at daily intervals, the results are presented as period averages. The daily output of feces was homogenized, and an aliquot of it kept under refrigeration. At the end of the period, the homogenized material was mixed and analyzed. The average daily excretion was obtained by dividing the total output by the number of days in the period.

TABLE 1

Composition and daily intake of essential and nonessential amino acid mixture

Component	Daily intake	Nitrogen content
	gm	*gm*
Essential amino acids		
L-Isoleucine	2.10[1]	0.224
L-Leucine	3.30	0.353
L-Lysine	2.40	0.460
L-Methionine	3.30	0.310
L-Phenylalanine	3.30	0.280
L-Threonine	1.50	0.176
L-Tryptophan	0.40	0.055
L-Valine	2.40	0.287
L-Histidine	0.75	0.203
Nonessential amino acids		
L-Arginine	11.78	3.790
L-Glutamic acid	7.98	0.760
L-Na-glutamate	11.34	0.939
Glycine	22.42	4.163
Total		12.000

[1] In experiment 2, isoleucine was adjusted to 1.5 gm, keeping the total nitrogen at a constant level by adding an isonitrogenous amount of glycine.

Results of experiment 1. Subject Y. D. (height, 159.1 cm; weight, 45.4 kg) received the amino acid mixture, amounting to 12 gm of total nitrogen (table 1). The daily energy intake was 55 Cal. per kg of body weight (not including calories derived from the amino acid mixture). The results obtained are presented in figure 1 and table 2. As anticipated, a positive nitrogen balance was established, indicating that 2.1 gm of isoleucine was sufficient to maintain the needs of this subject. Following this, the level of isoleucine intake was reduced to 0.3 gm, maintaining a constant nitrogen level by the substitution of nonessential amino acids. The nitrogen balance promptly became negative by 10%

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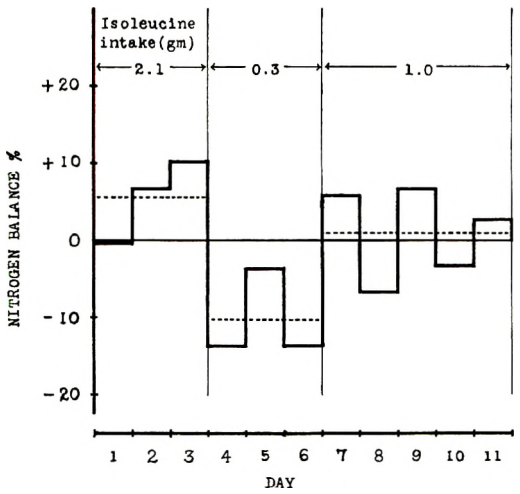

Fig. 1 Isoleucine intake and nitrogen balance of subject Y. D. The dotted line denotes the mean of levels of nitrogen balance at each period.

of the intake. Then isoleucine was supplied at a level of 1.0 gm, and a positive nitrogen balance was attained. Throughout the experiment, body weight and excretion of creatine and creatinine maintained a constant level (table 2). However, the excretion of creatine after taking the amino acid mixture increased more markedly as compared with that after consuming the normal diets. Rose et al. ('50) observed creatine in the urine of their subjects almost invariably, contrary to its absence in the urine of the normal male adult, and attributed their findings to the superior accuracy of the photoelectric method. We believe, however, that this might have been due to consuming the amino acid mixture rather than to the superiority of the method. By ingesting the amino acid mixture, subject Y. D. excreted the urinary creatine in larger amounts with no accompanying increase of creatinine, as compared with that when being fed with normal diets at his home. However, no explanation can be offered for this.

Subject H. G. (height, 147.2 cm; weight, 33.0 kg; daily energy intake, 46 Cal. per kg) received the amino acid mixture including 2.1 gm of isoleucine, and maintained a positive nitrogen balance, as in the case of Y. D. Following this, the level of isoleucine intake was decreased to 0.3 gm and the nitrogen balance became negative. Then by increasing the intake level of isoleucine to 1.0 gm, a positive nitrogen balance was attained.

Subject T. G. (height, 142.7 cm; weight, 27.8 kg; daily energy intake, 56 Cal. per kg) also maintained positive nitrogen balance with a level of 2.1 gm of isoleucine but not with 0.3 gm. Following this observation, the subject acquired a respira-

TABLE 2

Nitrogen balance and urinary excretion of creatine and creatinine at different levels of isoleucine intake

Subject	Period	Days	Body weight	Daily isoleucine intake	Daily N intake	Nitrogen balance	Average daily urinary excretion	
							Creatinine	Creatine
			kg	*gm*	*gm*	%	*mg*	*mg*
Y.D.							1026[1]	28[1]
	Preliminary	3	45.5	2.10	12.16	—	—	—
	1st	3	45.8	2.10	12.16	+5.6	986	214
	2nd	3	45.3	0.30	12.16	−10.3	1028	209
	3rd	5	45.6	1.00	12.16	+1.0	962	179
H.G.							710[1]	124[1]
	Preliminary	3	33.0	2.10	12.11	—	—	—
	1st	3	32.9	2.10	12.11	+8.3	593	306
	2nd	3	32.9	0.30	12.11	−11.9	714	286
	3rd	5	32.8	1.00	12.11	+1.8	669	192
T.G.							585[1]	164[1]
	Preliminary	3	27.8	2.10	12.11	—	—	—
	1st	3	27.7	2.10	12.11	+4.6	618	226
	2nd	3	27.5	0.30	12.11	−4.3	662	228

[1] Average of urinary excretion for two days, when subjects were being fed ad libitum at their homes.

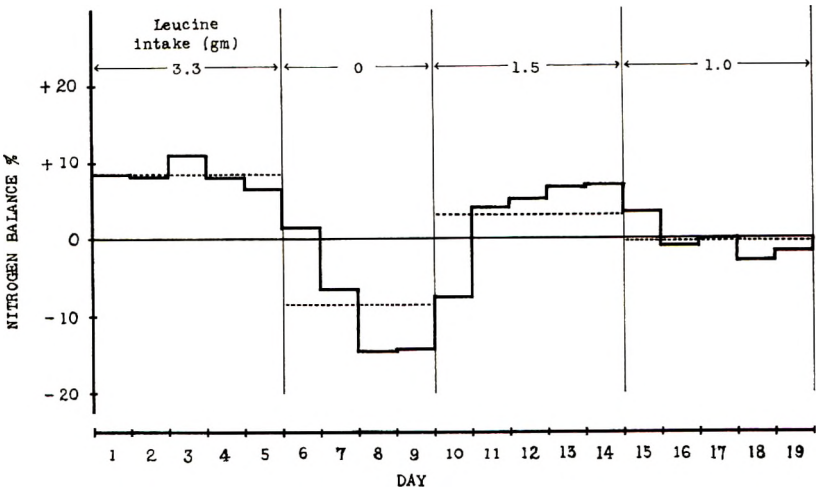

Fig. 2 Leucine intake and nitrogen balance of subject O. G. The dotted line denotes the mean levels of nitrogen balance at each period.

tory infection and could not be continued in the experiment.

Experiment 2. Three healthy boys, 11 years old, served as subjects for 23 days. The experimental method was the same used in the previous experiment, except that for three days preceding the administration of the amino acid mixture, a normal diet was given which was isonitrogenous to the mixture and consisted of the same menu throughout the three days. Furthermore, isoleucine in the mixture was adjusted to 1.5 gm. Since the possibility must be considered that children other than those tested may have still higher needs, we used isoleucine at a level of one-and-one-half times the amount which seemed to bring about a positive balance. The urine was analyzed not only for total nitrogen, creatine and creatinine, but also for riboflavin. Basal metabolism was also determined.

Results of experiment 2. Subject O. G. (height, 152.8 cm; weight, 37.0 kg; basal

TABLE 3

Nitrogen balance and urinary excretion of creatine, creatinine and riboflavin at different levels of leucine intake

Subject	Period	Days	Body weight	Daily leucine intake	Daily N intake	Nitrogen balance	Average daily urinary excretion		
							Riboflavin	Creatinine	Creatine
			kg	*gm*	*gm*	%	γ	*mg*	*mg*
O.G.								943[1]	141[1]
	1st	5	37.8	3.30	12.11	+8.4	531	912	215
	2nd	4	37.7	0	12.11	−8.5	749	877	445
	3rd	5	37.9	1.50	12.22	+3.2	725	925	411
	4th	5	37.2	1.00	12.22	−0.4	—	907	383
T.T.								624[1]	11[1]
	1st	5	28.6	3.30	12.11	+6.4	824	655	196
	2nd	4	28.1	0	12.11	−12.0	1050	675	250
	3rd	5	28.2	1.50	12.22	+4.7	926	659	216
	4th	5	27.8	1.00	12.22	−1.6	—	646	144
H.N.								683[1]	268[1]
	1st	5	27.0	3.30	12.11	+5.7	470	641	379
	2nd	4	26.4	0	12.11	−5.7	797	632	431
	3rd	5	26.6	1.50	12.22	+3.4	635	667	396
	4th	5	26.3	1.00	12.22	+6.0	—	656	368

[1] Average of urinary excretion for two days, when subjects were being fed ad libitum at their homes.

metabolism, 1171 Cal. per day) after taking the normal diet for three days, received the amino acid mixture listed in table 1. The daily energy intake was 56 Cal. per kg of body weight (not including calories derived from the amino acid mixture). This produced a positive nitrogen balance. On the 6th day, leucine was excluded from the amino acid mixture, keeping the total nitrogen at a constant level by adding an isonitrogenous amount of nonessential amino acid. The subject came into negative nitrogen balance. At this time he showed apparent symptoms of nervous irritability and failure in appetite. The excretion of riboflavin increased during the period when leucine was excluded (table 3). Then, with again adding leucine, at a level of 1.5 gm, the subjective symptoms disappeared and a positive nitrogen balance ensued. However, the nitrogen balance became negative again at a level of 1.0 gm of leucine (table 2 and fig. 2).

With the other two subjects, T. T. (height, 140.6 cm; weight, 28.5 kg; basal metabolism, 997 Cal. per day; daily energy intake, 50 Cal. per kg) and H. N. (height, 135.3 cm; weight, 26.5 kg; basal metabolism, 1194 Cal. per day; daily energy intake, 56 Cal. per kg), the observations were similar, except that H. N. definitely maintained a positive nitrogen balance at a level of 1.0 gm.

DISCUSSION

Since no studies have been made on the isoleucine requirement of children of school age, our results are compared with those of adults and infants. Rose et al. ('55) observed that 0.7 gm of isoleucine was required for daily minimal needs of the adult or 10 mg per kg of body weight. For infants, Holt et al. ('59) observed a requirement of 90 mg per kg. The subjects studied in our present experiment maintained a positive balance with an intake level of 1.0 gm of isoleucine. With the amino acid mixture including 3.3 gm of leucine, as shown in table 3, a positive nitrogen balance was maintained. When leucine was excluded, the nitrogen balance gradually became negative, and symptoms such as nervous irritability and failure of appetite appeared. Then when the nitrogen balance was determined at a level of 1.5 and 1.0 gm of leucine, all boys maintained a positive balance at 1.5 gm, but not always at 1.0 gm. The basal metabolism determined at the end of each period remained at a constant level. According to Pollack and Bookman ('51), labile proteins, which include the flavoproteins, increase or decrease rapidly as the body shifts from a positive to a negative balance, respectively. The amount of riboflavin stored in conjunction with these proteins would then likewise change rapidly. Smith et al. ('59) reported that when riboflavin intake was held constant and the nitrogen balance became negative by decreasing the intake of protein, urinary riboflavin increased. When the nitrogen balance became negative by an exclusion of amino acid, or of leucine, the excretion of riboflavin increased as in the case of protein. Creatinine was excreted at a constant level throughout the experimental period. Urinary creatine was also excreted in a large amount.

CONCLUSIONS

The minimal requirements of isoleucine and leucine were determined for 6 boys of school age by the use of the nitrogen balance method. From the results obtained it was estimated that children of 11 years of age require 1.0 gm of isoleucine and 1.5 gm of leucine as the daily minimal needs, or 30 and 45 mg per kg of body weight, respectively.

The children excreted a larger amount of creatine after receiving the amino acid mixture, as compared with that after consuming normal diets.

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Response of Cereal-Fed Guinea Pigs to Dietary Broccoli Supplementation and X-Irradiation

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The presence in cabbage of a substance which reduces the radiosensitivity of guinea pigs fed a basal diet of oats and wheat bran was originally reported by Lourau and Lartigue ('50) and confirmed by Duplan ('53). Later, another member of the *Brassicaceae* family, broccoli, was shown to be an even more effective supplement (Spector and Calloway, '59). The present study was undertaken to determine in what way animals fed broccoli differed from those not receiving the supplement and which of the responses subsequent to radiation exposure were modified, as a means of elucidating the mechanism responsible.

PROCEDURE

Young, male, albino guinea pigs of the Aristocratic strain weighing 250 to 350 gm, were held for a two-week adjustment period upon receipt from a local supplier. During this period all animals received ad libitum a diet consisting of 50% of whole field oats and 50% of pure wheat bran and a solution of sodium ascorbate (75 mg per 100 ml) in lieu of drinking water. Four animals were killed on receipt and 4 at the end of the standarization period. Two dietary groups were then designated at random: one group (1) continued to receive the same diet and the second (2) was given a supplement of 50 gm of raw broccoli daily. This treatment was followed for two weeks before and after total-body exposure to 400 r x-radiation.[2] Radiation factors were: 180 kv, 15 ma, no filtration added, 21 to 22 r per minute, 105 cm target distance. Six animals, three from each diet, were irradiated simultaneously in a horizontal beam, half of the dose being applied to each side of the body.

Control animals were killed on the day of irradiation. All other controls were subjected to all treatments other than administration of the radiation dose. After irradiation, certain nonirradiated animals were fed an amount of food equivalent to the average food consumption of the irradiated animals of the same diet group each previous day; these were designated "restricted controls." All others were fed ad libitum. Randomly selected animals were killed one, three, 5, 7, 9 and 14 days after irradiation. Mortality experience was such that no irradiated, group 1 animals survived until the 14th day.

Food intake was recorded daily and body weight intermittently throughout the experiment. Animals were housed individually in a controlled environment (26 ± 2°C), and all reasonable precautions were taken to assure a clean environment. However, upper respiratory infection was a prominent complication.

Tissues were obtained for histologic and biochemical examination following sodium pentobarbital anesthesia (50 mg per kg) and exsanguination.[3] All histologic and hematologic methods conformed to usual laboratory practices.

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[1] This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned no. 2062 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

[2] Irradiation was carried out at the Argonne National Laboratories.

[3] Histologic studies were conducted by the Hektoen Institute for Medical Research.

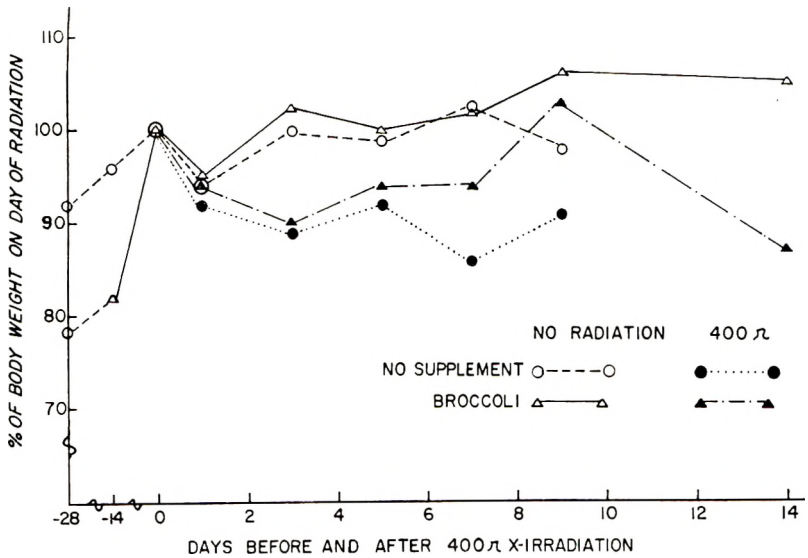

Fig. 1 Body weight of guinea pigs, expressed as percentage of weight on the day of irradiation. Mean body weights on that day were: no supplement, 318 gm (1); broccoli supplement, 367 gm (2).

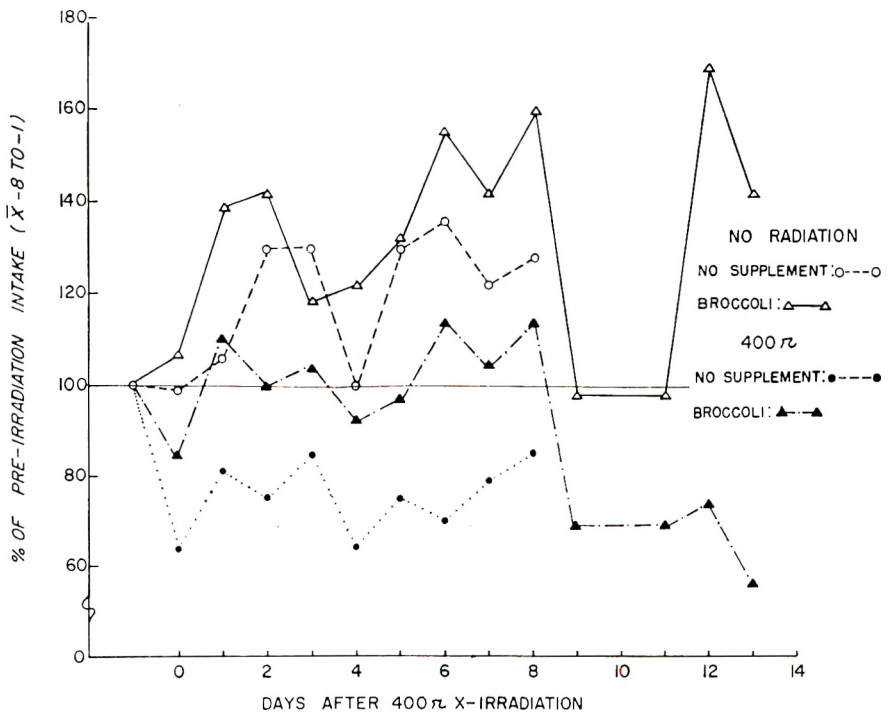

Fig. 2 Ad libitum food intake following irradiation, expressed as percentage of the average eaten daily for one week prior to exposure. These amounts were: basal 17.4 gm, no supplement (1); basal 13.8 gm plus broccoli 49 gm (2).

For simplicity, the tables indicate a single mean value for all control animals over all time intervals. Statistical comparisons, however, were made between control and irradiated animals killed on any given day. Treatment effects were assessed by analysis of variance (Brownlee, '53).

RESULTS

Body weight and food intake data are presented graphically in figures 1 and 2, as percentages from a standard point. This scheme was used, because of the changing population at each time interval, to avoid artifacts induced by removing extreme values. Before irradiation, superior weight gain was achieved as a consequence of broccoli supplementation. Slight weight loss was recorded 24 hours after irradiation in both groups which reflected handling effects rather than irradiation, as the same change was apparent in the control population. Control animals regained this loss promptly and held weight constant for the remainder of the test. In the irradiated groups an additional small loss occurred by the third day, followed by an essentially steady state. Food intake was depressed in both irradiated groups, but to a lesser degree in group 2. At necropsy, a general absence of body fat and in some cases a gelatinous degeneration of the perirenal fat was seen most frequently in group 1.

Various manifestations of the radiation hemorrhagic syndrome were observed in animals from both groups sacrificed 5 days post-irradiation and thereafter (table 1). These consisted primarily of multiple diffuse intradermal hemorrhages, subserosal and mucosal hemorrhages of the gastrointestinal tract, and hemorrhages of the myocardium, the skeletal muscles, the meninges and the medullary contents of the long bones. These were observed to occur with about equal frequency and severity in both dietary groups, and presented no remarkable microscopic features. Patches of atelectasis and consolidation were seen in the lungs of some irradiated and nonirradiated animals of both dietary groups. No bacteriologic studies were conducted.

The nonirradiated controls of the bran and oats diet group presented a number of striking morphologic changes which were also evident in the irradiated members of this group. A frequent observation was the presence of a greatly distended gallbladder containing bile in which an amorphous sediment was present. Microscopic examination of the sediment indicated it to be composed of cellular detritus and mucus. This condition was seen only infrequently in the supplemented group.

Among the animals in group 2, both irradiated and nonirradiated, multiple subcapsular yellow nodules were seen on the surfaces of the liver. Microscopically these were seen as areas of focal fatty changes. The livers of group 2 animals were also significantly larger than those of group 1, both irradiated and control (table 2). Irradiation resulted in diminished liver weight in both groups, probably as a result of voluntray food restriction, since comparable changes were seen in the restricted controls. This weight loss was recovered by the 9th day in group 1 and by the 14th in group 2. Histopathologic studies revealed reduced liver glycogen content of animals in group 1 as compared with those in group 2. The group 1 animals had liver glycogen stores approximately the same as those of the initial controls. In addition, on microscopic examination moderate intralobular cholangiole proliferation and some areas of hydropic degeneration were seen in animals from both groups.

Liver vitamin A content was 4 times as great in controls of group 2 as in group 1, 17 I.U. as compared with 4 I.U. (table 2). Values recorded in irradiated animals did not differ significantly from their own diet controls. The low levels of group 1 reflect a marked drop from animals as received from the supplier.

In a number of animals in group 1 the adrenals developed a grayish color. In some, this condition was so marked as to make it impossible to distinguish between the cortical and medullary portions on transverse section. Histopathologic examination, however, failed to reveal any differences between the adrenals of the two groups. No significant difference in adrenal size was evident due to diet. Following irradiation, adrenal size dropped significantly in group 1 at day 5 but group 2 remained constant until day 14 when enlargement was noted.

TABLE 1

Gross pathologic observations

	Percentage incidence on days after irradiation as noted[1]												
	No supplement						Broccoli supplement						
	Control	1	3	5	7	9	Control	1	3	5	7	9	14
Radiation injury													
Multiple hemorrhages in													
Myocardium	—	—	—	—	—	17	—	—	—	—	—	33	—
GI tract (mucosa-serosa)	—	—	—	—	50	100	—	—	—	—	17	83	—
Intradermal	—	—	—	—	—	83	—	—	—	—	—	67	14
Parenchyma of spleen	—	—	—	—	—	—	—	—	—	—	—	17	—
Lymph nodes	4	—	—	—	—	50	4	—	—	—	—	67	57
Meninges	—	—	—	—	—	17	—	—	—	—	—	—	29
Lungs	18	—	—	—	17	50	8	—	—	—	33	50	72
Epididymites	—	—	—	—	33	17	—	—	—	—	33	50	57
Skeletal muscles	—	—	—	—	—	17	—	—	—	—	33	50	—
Epiphyses of bones	—	—	—	—	—	—	—	—	—	—	—	17	—
Medullary cavities	—	—	—	—	—	33	—	—	—	—	—	33	—
Edema	—	—	17	—	17	—	—	—	—	—	—	—	—
Infection													
Pneumonia	23	—	17	17	17	17	15	—	—	33	17	33	14
Lymphadenitis	4	—	—	—	—	—	—	—	—	—	—	—	—
Multiple liver abscesses	—	—	—	—	—	—	—	—	—	—	—	—	29
Nutritional or metabolic pathology													
Distention of gallbladder with sediment in bile	13	—	—	33	67	17	—	—	17	—	17	17	—
Gross fatty changes (liver)	4	17	17	—	—	—	40	50	33	50	50	33	29
Gelatinous fat degeneration	9	—	—	—	17	50	—	—	—	—	17	—	—
Congenital defects													
Rectal prolapse	4	—	—	—	—	—	—	—	—	—	—	—	—
Gallbladder absent	—	—	—	—	17	—	—	—	—	—	—	—	—
Unknown etiology													
Gray discoloration adrenals	9	—	—	17	50	17	—	—	—	—	—	—	—

[1] Total N: diet 1 controls=22; diet 2 controls=25; days 1, 3, 5, 7 and 9 both diets=6; day 14=7.

TABLE 2

Tissue characteristics of control and irradiated guinea pigs

	Initial control	Standardized control	Diet	Controls		Days after irradiation					
				Fed ad lib.	Restricted-fed	1	3	5	7	9	14
Number	4	4	1	14	8	6	6	6	6	6	
			2	15	10	6	6	6	6	6	6–7
Liver weight, gm/100 gm body weight	3.72	3.20	1	3.94	3.43[c]	3.63	3.42	3.40	3.98	4.00	
			2	4.36[a]	4.23[a,c]	4.32[a]	4.26[a]	3.97[a]	3.87	4.19	4.92
Liver vitamin A content, I.U./gm	26	10	1	5	4	4	5	4	4	4	
			2	19[a]	14[a]	15[a]	24[a]	21[a]	16[a]	15[a]	26
Adrenal weight, mg/100 gm body weight	48	44	1	64	54[c]	64	63	53[b]	68	61	
			2	55	56	53	54[a]	55	56	56	69[b]
Testes weight, mg/100 gm body weight	178	143	1	82	108	113	151	106[b]	89	107	
			2	238[a]	214[a]	204[a]	210	240[a]	150[a]	173	164[b]
Spleen weight, mg/100 gm body weight	194	123	1	137	125[c]	104[b]	112	99[b]	102	91[b]	
			2	151	143[c]	119[a,b]	110	103[b]	120[a]	93[b]	110[b]
Heart weight, mg/100 gm body weight	455	369	1	331	351	358	332	326	378	319	
			2	371	390	388	381	352	351	343	442
Kidney weight, gm/100 gm body weight	0.96	0.98	1	0.97	0.95	1.00	0.98	0.96	1.10[b]	1.05	
			2	0.92[a]	0.92	0.89	0.93	0.89	0.86[a]	0.89[a]	1.13[b]
Serum nonprotein N, mg/100 ml	42	40	1	45	41	59	49	41	48	45	
			2	41	38	53	40	38	46	43	48
Total leukocytes/mm^3			1	2471	3641	1700	1080	467[b]	508[b]	546[b]	
			2	3680	3920	1567[b]	1200[b]	438[b]	434[b]	500[b]	300[b]
Erythrocytes, million/mm^3			1	4.90	4.78	4.94	4.99	4.65[b]	4.29	4.18[b]	
			2	4.90	4.98	5.06	4.62	3.74[b]	4.18[b]	3.27[b]	1.73[b]
Hematocrit, %			1	42	43	42	42	41	40	40	
			2	43	45	42	40	39	41	30[b]	17[b]
Hemoglobin, gm/100 ml			1	14.1	14.7	13.9	15.1	14.1	13.6	14.2	
			2	14.1	14.5	14.0	13.7	13.0[b]	13.4	10.3[a,b]	5.8[b]

[1] Letters refer to statistically significant differences ($P < 0.05$): a indicates diet 1 vs. diet 2 on any given day, i.e., diet effect; b indicates irradiated vs. nonirradiated of a given diet on a given day, i.e., radiation effect; c indicates ad libitum-fed vs. restricted-fed controls of a given diet, i.e., food restriction effect.

Most striking of the changes observed was the atrophic appearance of the testes and secondary sex organs of the animals in group 1. Histopathology revealed a decrease in the size of the semiferous tubules as compared with initial and standardized controls, but without differences in spermatogenic activity. Testes weight was significantly greater in group 2 animals, both irradiated and control. Following irradiation, testes weight was diminished significantly by the 9th day in group 1 but not until the 14th day in group 2. Spermatogenesis was depressed in both groups after irradiation.

Spleen weight did not vary significantly between controls of the two diet groups. Although the weight of this organ was reduced in restricted controls, a much more pronounced diminution was apparent in irradiated animals, a phenomenon described by others (Bloom and Bloom, '54). Morphologic changes also followed the typical pattern of lymphoid degeneration.

Although heart weight was greater and kidney weight less on the average in group 2 than in group 1, the differences were of marginal significance. No demonstrable change in heart mass occurred following irradiation. In group 1, kidney size remained essentially constant until the 7th day, when an increase was noted; on days 7 and 9 kidney size was significantly greater in group 1 than in 2. A comparable increase was observed in group 2 by the 14th day. No analytical data bearing on the composition of the increased weight were obtained. There was a transient increase in serum nonprotein nitrogen content (Δ 15 mg/100 ml) 24 hours after irradiation but whether this is related to kidney function is unknown.

Leukocyte counts[4] showed enormous variation in the control population and no significant effect of diet. A precipitous fall in white cells was seen at 24 hours after irradiation and values were maximally reduced by the 5th day. No recovery was evident in the period of study in either group. Histologically, the lymph nodes of the irradiated animals in group 1 appeared to have suffered more extensive injury and undergone a greater degree of disorganization than those in group 2. The regenerative process in the lymph nodes of animals in group 2 was confined to the germinal centers of the cortex where it proceeded in an orderly diffuse pattern from the 5th day post-irradiation onward. In group 1 the regenerative processes were most active in the medullary cords rather than the cortex.

Although the pathologist reported depression of bone marrow in some group 1 controls, erythrocyte count, hematocrit and hemoglobin values were identical with those in group 2. Hypoplasia was seen in the bone marrow as early as day 1 and was marked at days 3, 5 and 7. Red cell counts began to fall about the 5th day, attaining lower levels in group 2 than in 1. The lowest values recorded were those of day 14 when counts ranged from 1:30 to 2.58 million per mm^3, packed cell volume from 9 to 28%, and hemoglobin from 3.6 to 8.5 gm per 100 ml.

DISCUSSION

The radiation dose administered is, in our experience, about $LD_{60/30}$ for groups of animals fed commercial guinea pig feed. Thirty-day mortality in guinea pigs given bran and oats plus broccoli is normally 30 to 40% and in those given bran and oats alone is 95 to 100% (Spector and Calloway, '59). Neither gross nor microscopic features of the radiation-induced changes reported here are unusual for acute exposure in this dose range.

The profound differences between the nonirradiated animals of the two groups require comment. Several nutritional imbalances exist in the bran and oats basal diet (see table 3). The diet is devoid of vitamin A and remarkably low in protein, riboflavin, calcium, sodium and the halogens. Pantothenic acid, folic acid and iron levels are marginal and phosphorus content is excessive. Supplementation with broccoli improves the dietary with respect to each of these factors and corrects for all vitamin insufficiences. The sediment observed in the gallbladders of group 1 animals is a recognized manifestation of

[4] All blood components were influenced to an unknown degree as a consequence of pentobarbital administration. If one accepts the assumption that both groups were affected to the same degree, then the data have comparative but not absolute value. No evidence bearing on the validity of this assumption is at hand.

TABLE 3

Comparison of nutrient composition of the diets fed with commercial guinea pig chow

		Commercial feed	Bran-oats	Bran-oats + broccoli[1]
	per 100 gm			
Nitrogen	gm	3.34	2.08	2.58
Lipid	gm	5.29	2.74	2.47
Ash	gm	7.61	4.90	5.79
α-Tocopherol	mg	3.2	3.5	7.7
β-Carotene	μg	390	tr.	2000
Thiamine	mg	0.676	0.585	0.707
Riboflavin	mg	0.747	0.155	0.563
Niacin	mg	6.6	13.0	12.2
Pyridoxine	mg	0.74	0.79	1.07
Ca-pantothenate	mg	4.31	2.34	5.11
Folic acid	μg	290	128	214
Biotin	μg	42	45	56
Vitamin B_{12}	μg	2.8	0.2	< 0.2
Choline Cl	mg	241	222	285
Inositol	mg	245	459	465
Calcium	gm	1.52	0.30	0.51
Phosphorus	gm	0.57	1.07	1.02
Sodium	mg	177	5[2]	155
Potassium	gm	1.70	1.07	1.48
Magnesium	mg	310	320	368
Zinc	mg	4.7	3.6	3.6
Manganese	mg	23	21	18
Iron	mg	17	8	10
Copper	mg	0.7	0.7	1.3
Selenium	μg	< 2	< 2	*ca.* 12
Molybdenum	μg	150	100	141
Cobalt	μg	5	5	9
Aluminum	mg	1.5	2.3	2.8
Chlorine	mg	330	26	133
Fluorine	mg	1.59	< 0.2	*ca.* 0.24
Bromine	mg	7.5	< 0.4	*ca.* 5.9
Iodine	mg	0.09	0	0.59
Sulfur	mg	248	156	396

[1] Calculated on the basis of 20% of broccoli dry solids with 80% of the bran-oats mixture. This ratio is the best estimate of proportional consumption based on 7 separate experiments.
[2] The drinking solution of sodium ascorbate provides 27 mg/100 ml in addition.

avitaminosis A in this species (Erspamer, '38) and vitamin A insufficiency is also reflected in the reduced content of vitamin A in the livers of this group. Whether the other differences noted in the biliary system, i.e., increased liver size and minimal fatty changes in group 2, are a consequence of nutritional status or due to specific positive effects of broccoli feeding cannot be determined from the data at hand.

The involution of the gonads and accessory sex organs, the retardation of growth, reduced liver glycogen content and the lack of body fat seen in group 1 indicate a hormonal derangement of dietary etiology. These changes closely resemble those of the pseudohypophysectomy described by Mulinos and Pomerantz ('40), induced by chronic food restriction. In the present experiment, total dry solids intake differed by about one gram per day, on the average, prior to irradiation. Therefore, the developmental differences may be ascribable primarily to qualitative restriction, as well as to marginal quantitative limitation. The rapid appearance of these changes indicates the sensitivity of the guinea pig to nutritional factors. The gonads and particularly the accessory sex organs are sensitive indicators of endocrine function. It has been well established that a number of interrelationships exist between nutrients and endocrine glands, their secretions, and the responses of target organs or tissues to hormonal stimulation. Evidence exists that certain nutritional requirements are increased dur-

ing conditions of stress. The changes observed in the nonirradiated, ad libitum-fed animals of group 1 indicate them to be poorly equipped to respond to stress of any kind.

An attractive hypothesis for the greater radiosensitivity of the unsupplemented group, then, lies simply in nutritional deficiency. Both chronic caloric restriction[5] and vitamin A deficiency (Ershoff, '52) have been shown to lower resistance to X-irradiation. However, preliminary studies have been conducted in which the quality of the bran-oats diet was improved with respect to protein, vitamins and the major minerals, alone or in consonance. Beneficial effects were noted in growth but radiosensitivity was not consistently reduced. Studies are in progress to determine what contributions to the bran-oats diet made by the broccoli supplement enable guinea pigs to withstand radiation insult.

SUMMARY

Young, male, guinea pigs were fed a basal diet of bran and oats, with or without a daily supplement of 50 gm of raw broccoli, for two weeks before and after exposure to 400 r whole body x-radiation. Animals were killed sequentially for histologic and hematologic examination. Provision of broccoli resulted in improved nutriture, larger livers with higher stores of vitamin A and mimimal fatty changes, and superior gonadal development. Following irradiation in both groups, decrements in body weight and food intake as well as the characteristic events of the radiation syndrome were seen. Broccoli supplementation of the cereal diet generally reduced the degree of distortion or delayed its appearance.

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