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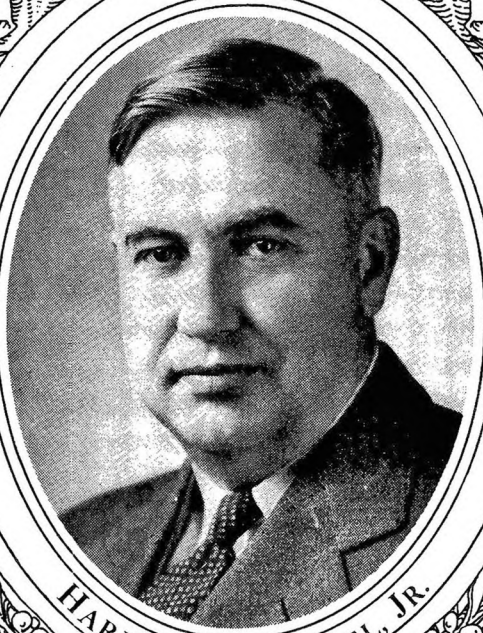

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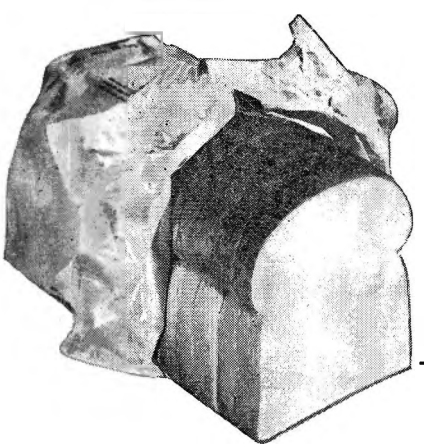

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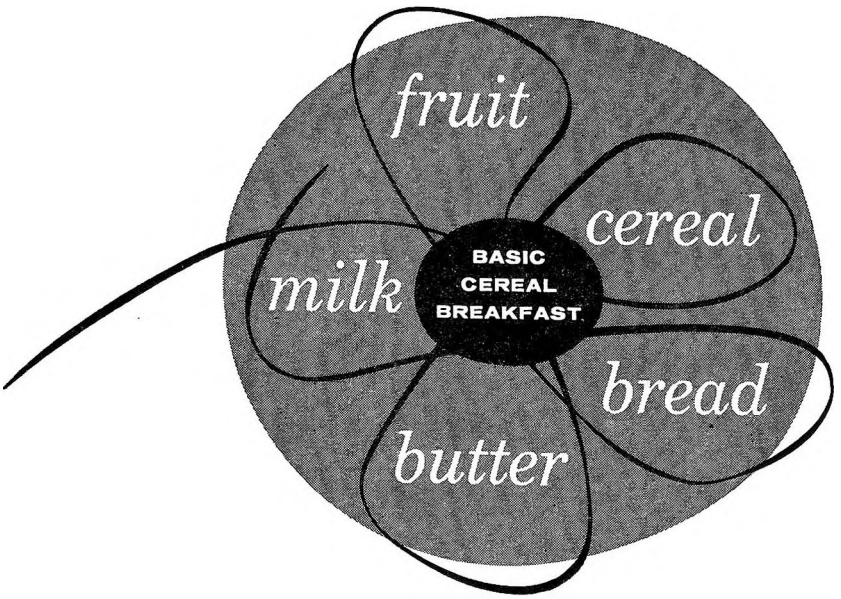

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Milk, nonfat (skim), 1 cup,
black coffee

Nutritive value of basic cereal breakfast pattern

CALORIES	502	VITAMIN A	600 I.U.
PROTEIN	20.5 gm.	THIAMINE	0.46 mg.
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Note: To further reduce fat and cholesterol use skim milk on cereal which reduces Fat Total to 7.0 gm. and Cholesterol Total to 16.8 mg. Preserves or honey as spread further reduces Fat and Cholesterol.

Bowes, A. deP., and Church, C. F.: Food Values of Portions Commonly Used. 8th ed. Philadelphia: A. deP. Bowes, 1956.
Cereal Institute, Inc.: The Nutritional Contribution of Breakfast Cereals. Chicago: Cereal Institute, Inc., 1956.
Hayes, O. B., and Rose, G. K.: Supplementary Food Composition Table. J. Am. Dietet. A. 33:26, 1957.
Cereal Institute, Inc.: A Summary of the Iowa Breakfast Studies. Chicago: Cereal Institute, Inc., 1957.

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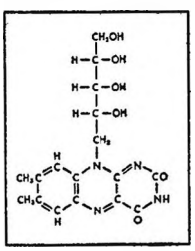
The Vital Stor

A Quick History. Independent investigators, working separately to unlock several of nature's doors, sometimes open up unsuspected relationships. This happened with vitamin B_2.

Investigations. About 25 years ago, several groups, notably Warburg's, were investigating a "yellow enzyme" obtained from yeast. Almost simultaneously other investigators were studying a food factor that aided growth of laboratory animals.

What they found. Proceeding with chemical analysis of this growth factor, the team of Kuhn, György, and Wagner-Jauregg noted a relationship between the growth-producing agent and the "yellow enzyme." Their findings, and those of other researchers along similar lines, were published in 1933. Eventually, riboflavin and an essential part of the yellow enzyme were found to be identical and the unity of an essential nutrient and cellular metabolism was established.



Isolation of pure riboflavin was achieved by Kuhn and his co-workers, and by Ellinger and Koschara, in 1933.

Nomenclature. Known in the United States as riboflavin, this vitamin has also been called lactoflavin, ovoflavin, hepatoflavin, and vitamin G.

SYNTHESIS

By 1935, two eminent chemists, working separately, had synthesized riboflavin, practically in a dead heat. Prof. Paul Karrer of the University of Zurich, a collaborator of the Hoffmann-La Roche Laboratories, produced the first successful synthesis. Five weeks later Richard Kuhn of Germany announced his synthesis of the vitamin. Prof. Karrer subsequently shared the Nobel Prize in Chemistry for his work in vitamins and carotenoids.

The Karrer synthesis forms the basis for chemical processes in widespread use today by Hoffmann-La Roche and other leading manufacturers throughout the world. Riboflavin is also manufactured today by fermentation methods.

CHEMICAL AND PHYSICAL PROPERTIES

Riboflavin is yellow, slightly water-soluble with a greenish fluorescence and a bitter taste. Its empirical formula is $C_{17}H_{20}N_4O_6$. Vitamin B_2 produced by the Roche process is identical in every way with that occurring in nature.

How does vitamin B_2 work? Riboflavin is a vital part of nature's chain of reactions for utilization of carbohydrate energy. It has been found to be a constituent of many enzy systems and is thus intimately connected with life proces It is probably required by the metabolic processes of every animal and bird as well as by many fishes, insects and lower forms of life. (In certain animals, however, the requirement may be synthesized by bacteria within the intestine.)

In the cells riboflavin goes to work attached to a phosphate group. This substance, known as riboflavin-5'-phosphate or flavin mononucleotide, may in turn be attache still another essential substance, adenylic acid, forming fl adenine dinucleotide. Either nucleotide then is attache protein, thereby forming an enzyme, and takes its par oxidation-reduction reactions.

Requirements in Human Nutrition. As we have seen, v min B_2 is essential to life. We have no special storage org in our bodies for this vitamin, although a certain leve maintained in various tissues, with relatively large amou found in the liver and kidneys.

MEASURING METHODS

In the beginning, riboflavin activity was described in "Bo quin-Sherman units" and requirements were thought to very small. Subsequent research show otherwise. Milligrams of weight beca the unit and the Food & Drug Admir tration of the U. S. Dept. of Heal Education & Welfare established (J 1, 1958) a minimum daily requirem of 1.2 mg. of riboflavin for all persons 12 or more ye old. For infants it is 0.6 mg. These requirements are signed to prevent the occurrence of symptoms of ribofla deficiency disease. The minimum daily requirement for t vitamin for children from 1 to 12 years is 0.9 milligra

Recommended allowances. The Food & Nutrition Bo of the National Research Council has recommended the f lowing daily dietary allowances of riboflavin, expressed milligrams. These are designed to maintain good nutrition healthy persons in the U. S. A.

Men		1.6	
Women		1.4	
"	(3rd trimester of pregnancy)	2.0	
"	(Lactating)	2.5	
Infants,	1-3 months	0.4	
"	4-9 "	0.7	
"	10-12 "	0.9	
Children,	1-3 years	1.0	
"	4-6 "	1.2	
"	7-9 "	1.5	
		Boys	Girls
Adolescents,	10-12 years	1.8	1.8
"	13-15 "	2.1	2.0
"	16-20 "	2.5	1.9

# of VITAMIN B_2 *by* Science Writer

(Riboflavin)

Deficiencies of vitamin B_2 appear in several ways in human beings. The eyes, the skin, the nerves, and the blood show the effects of too little riboflavin. Laboratory animals have demonstrated that a riboflavin-deficient diet can cause death of adults and can slow or stop growth in the young. Female animals, deprived of riboflavin in the diet, may produce offspring with congenital malformations.

Medical uses. To overcome and control deficiencies in human beings, physicians have pure riboflavin available for administration by injection or orally, by itself or with other "B" vitamins or multi-vitamin-mineral combinations.

How do we get our daily riboflavin? Vitamin B_2 has wide distribution throughout the entire animal and vegetable kingdoms. Good sources are milk and its products, eggs, meats, legumes, green leaves and buds. Whole-grain cereals have significant but not large amounts of riboflavin.

ADDITION TO FOODS

Cereal foods play a large part in our diet. To produce the white flour almost all of us want, millers are obliged to remove parts of the wheat that contain much of the grain's riboflavin and other nutrients. In addition, cereal grains are not rich sources of riboflavin. Millers meet this problem by *enriching* the grain foods for which federal standards exist with vitamins B_1, B_2, niacin and the mineral iron. In the case of vitamin B_2, however, they do more than *restore* the processed food to its natural riboflavin level; they *fortify* the food with enough of this essential vitamin to make it nutritionally more valuable than it was in nature.

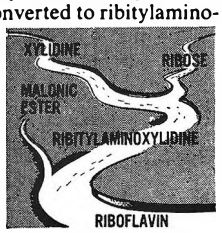
Acting to protect the good health of millions of Americans, bakers and millers adopted *enrichment* of white bread and white flour in 1941. Since that time, other foods, such as macaroni products, corn meal and grits, farina, pastina and breakfast cereals have had their food value increased by enrichment with pure riboflavin and other vitamins and minerals.

When enriching, fortifying or restoring, food manufacturers add the necessary quantity of riboflavin (and other vitamins and minerals) to the food during processing, so that the finished product meets federal, state, and territorial requirements or contributes to the consumer an amount of the vitamin that dietary experts believe significantly useful.

PRODUCTION

Prof. Karrer's synthesis of riboflavin was a laboratory success. Adapting the process to commercial production, however, demanded original thinking by chemists at Hoffmann-La Roche. The production of riboflavin by chemical synthesis requires the production of ribose, a rare sugar, at an early stage in the process. This special sugar must be made inexpensively if the synthesis is to be practical. Sugar chemistry is a difficult matter. In a brilliant piece of work, the Roche chemical experts developed a method to produce ribose on a commercial scale by an electrolytic process, thus overcoming a most troublesome problem. Subsequently, Roche chemists developed the first practical synthesis for riboflavin-5'-phosphate, identical with natural flavin mononucleotide.

Picture three streams joining to form a river and you have a simplified idea of the Roche process for synthesizing vitamin B_2. O-xylene and glucose are processed separately to form xylidine and ribose respectively. These are joined to form ribitylxylidine, which is then converted to ribitylaminoxylidine. Starting separately with malonic ester, which is processed through intermediate stages to alloxan, the third "stream" is then joined with ribitylaminoxylidine to form riboflavin. Purification occurs at each step of the synthesis. Riboflavin 'Roche' equals or exceeds U. S. P. standards.



By the tons. So efficient is the Roche process that pure riboflavin is produced *by the tons* for use in pharmaceutical products and processed foods. An interesting development by Roche is the production of riboflavin in different forms related to the method of end use. 'Roche' Regular riboflavin U. S. P. is especially useful in dry enrichment premixes, powdered dietary supplements, pharmaceutical tablets and soft gelatin capsules. 'Roche' Solutions type is preferred for the manufacture of solutions having low concentration. 'Roche' Riboflavin-5'-Phosphate Sodium is a highly and rapidly soluble riboflavin compound favored for all pharmaceutical liquid products and some tablets, lozenges, and capsules. It has a more pleasant taste than the bitter U. S. P. riboflavin.

This article is published in the interests of pharmaceutical manufacturers, and of food processors who make their good foods better using pure riboflavin 'Roche.' Reprints of this and others in the series will be supplied on request without charge. Also available without cost is a brochure describing the enrichment or fortification of cereal grain products with essential vitamins and minerals. These articles and the brochure have been found most helpful as sources of accurate information in brief form. Teachers especially find them useful in education. Regardless of your occupation, feel free to write for them. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 1956 Bourdon St., St. Laurent, P. Q.

EFFECTS OF THE ESTERIFICATION OF SUPPLEMENTAL CHOLESTEROL AND SITOSTEROL IN THE DIET [1]

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(Received for publication December 24, 1957)

Almost all of the cholesterol entering the intestine, whether in the bile or of dietary origin, is in the free state. Of the cholesterol absorbed, however, the majority appears in the thoracic duct lymph as esterified cholesterol. Vahouny and Treadwell ('57) have presented data suggesting that in the rat up to 90% of absorbed cholesterol is esterified between its introduction into the intestine and its appearance in the thoracic duct lymph. Daskalak' and Chaikoff ('55) fed C^{14}-labelled cholesterol to rats and observed that, regardless of the amount fed, approximately 70% of the labelled cholesterol recovered in the lymph was esterified.

The site of this esterification of cholesterol has not been clearly established. Pancreatic cholesterol esterase, and bile salts essential to its activity, are present in the intestinal lumen and the classical concept has been that the esterification of cholesterol occurs in the lumen prior to absorption (Peters and Van Slyke, '46). More recent studies (Pihl, '55), including the demonstration of a cholesterol esterase in the intestinal mucosa (Swell, Byron and Treadwell, '50), suggest that the

[1] This investigation was supported by research grants from the U. S. Public Health Service (H-1946), the American Heart Association, the Heart Association of Louisville and Jefferson County and Eli Lilly and Company. The authors are indebted to Drs. Robert E. Shipley, Herschel D. Porter and M. Korzenovsky of the Lilly Research Laboratory for the preparation of the esters employed and the determination of *in vitro* rates of hydrolysis.

intestinal mucosa rather than the lumen may be the site of esterification.

The first part of the present communication reports the effect of prior esterification of dietary cholesterol on its accumulation in serum and liver of the rat. The results suggest that cholesterol esters are not absorbed as such, but are first hydrolyzed in the intestinal lumen to the free sterol.

The second part of the study relates to the inhibitory effect of β-sitosterol, a poorly absorbed plant sterol (Gould, '55), on cholesterol absorption. Previous reports have indicated that sitosterol does interfere with cholesterol absorption in the rat (Hernandez et al., '53) and prevents the increase in liver cholesterol which otherwise occurs on a high-cholesterol diet (Best and Duncan, '56). The inhibition of cholesterol absorption by various esters of sitosterol has been compared to that of the free sterol. The results suggest that sitosterol esters also undergo hydrolysis in the intestine and that any inhibitory effect they exert on cholesterol absorption is due to the resultant free sitosterol.

METHODS

Male white rats [2] of approximately 300 gm weight were employed. Prior to the experimental period they were maintained on a balanced laboratory ration.[3] The animals were divided into groups of similar mean weight and were housed in mesh-bottom cages in an air conditioned animal room maintained at 25°C. Tap water was provided at all times. Each of the experimental diets was offered ad libitum for a period of 14 days. At the conclusion of the experimental feeding period, the animals were anesthetized with intraperitoneal amobarbital sodium. Blood was collected by cardiotomy and the liver removed and blotted. A weighed portion of each liver was placed in a tightly covered jar containing 3 ml per gram of liver of a 25% potassium hydroxide solution in 95% ethyl alcohol. The jars were placed

[2] Holtzman.

[3] Purina Laboratory Chow.

overnight in a water bath at 37°C. Serum cholesterol was determined by the method of Abell et al. ('52). To 1 ml of the liver digestant was added 5 ml of 95% alcohol. Petroleum ether extraction and determination of cholesterol were then performed in the same manner as for serum.

In those animals in which free as well as total liver cholesterol was determined, 1 to 2 gm of the liver was homogenized in acetone-ethanol in a Waring Blendor, and free and total cholesterol determined by the method of Sperry and Webb ('50).

Sitosterol is precipitated by digitonin and gives the Liebermann-Burchard color reaction; thus "cholesterol," as determined by the methods employed, would include to a great extent any sitosterol present in serum or liver.

Effect of feeding free cholesterol and various cholesterol esters. Seven groups of from 8 to 11 animals each were employed in this study. The basic diet of all groups consisted of commercial rabbit pellets [4] with 5% added cottonseed oil. The control group received this essentially cholesterol-free diet. To the basic diet of each of the remaining 6 groups one of the following was added: 1% cholesterol (M.P. 148 to 150°C); 1.11% cholesteryl acetate (M.P. 112 to 114°C); 1.27% cholesteryl benzoate (M.P. 142 to 146°C); 1.62% cholesteryl palmitate (M.P. 73 to 75°C); 1.69% cholesteryl stearate (M.P. 79 to 81°C); and 1.92% "cholesteryl oleate." The free cholesterol and various esters were dissolved in the warmed cottonseed oil prior to addition to the rabbit pellets. The esters, with the exception of the oleate, contained no free cholesterol as determined by digitonin precipitation. The "cholesteryl oleate" was incompletely esterified, and contained an excess of oleic acid; of the cholesterol present, 62% was esterified and 38% was in the free state. All the esters were added to the diet in such amount as to provide 1% cholesterol by weight.

The results are given in table 1. The addition of 1% cholesterol to the diet resulted in a marked increase in liver

[4] Purina Rabbit Pellets.

TABLE 1

Effect on serum and liver cholesterol of the rat of feeding free cholesterol and various cholesterol esters

NO. RATS	ADDED TO BASIC DIET	WEIGHT		SERUM TOTAL CHOLESTEROL	LIVER CHOLESTEROL	
		Initial	Gain		Free [1]	Total
	%	*gm*	%	*mg/100 ml*	*mg/100 gm*	*mg/100 gm*
8	None	297	10	74 ± 5 [2]	232 ± 16 [2]	298 ± 10 [2]
10	Free cholesterol, 1	304	11	86 ± 8	264 ± 25	1326 ± 315
11	Cholesteryl acetate, 1.11	304	11	82 ± 13	252 ± 19	1214 ± 478
8	Cholesteryl benzoate, 1.27	299	10	80 ± 9	231 ± 10	333 ± 48
8	Cholesteryl palmitate, 1.62	301	13	74 ± 5	217 ± 13	353 ± 40
8	Cholesteryl stearate, 1.69	299	13	81 ± 4	222 ± 10	334 ± 25
11	"Cholesteryl oleate," 1.92	302	11	76 ± 7	256 ± 21	1300 ± 218

[1] Liver free cholesterol determined in 4 animals in each group.

[2] Values given are the mean and standard deviation, estimated using (n — 1).

total cholesterol, the mean being significantly higher than in the control group ($P < 0.01$). Of the esters fed, the cholesteryl acetate and "cholesteryl oleate" resulted in elevation of liver cholesterol to essentially the same extent as did the free sterol. The increase in liver cholesterol was much less in the groups receiving the three remaining esters, the mean liver cholesterol in the groups receiving cholesteryl palmitate, stearate and benzoate being significantly lower than that of the groups fed free cholesterol or the acetate or oleate ($P < 0.01$). The mean concentration of free cholesterol in the liver, determined in 4 animals of each group, was relatively constant; the increment in liver total cholesterol resulting from cholesterol feeding was largely in the ester fraction.

Serum total cholesterol differed but little in the various groups. The maximum response, an increase of only 12 mg/100 ml, was exhibited by the animals receiving free cholesterol.

Effect of the addition to the diet of the cholesterol fed rat of free sitosterol and various sitosterol esters. Seven groups of from 4 to 8 animals were employed. The basic diet in this experiment consisted of a balanced laboratory ration [5] to which was added 5% cottonseed oil. One group of animals received the basic diet, unmodified. Cholesterol, 1% by weight, was added to the diets of the remaining 6 groups. In addition to the added cholesterol each of 5 groups received one of the following further dietary supplements: 5% beta-sitosterol (M.P. 133 to 135°C); 5.51% sitosteryl acetate (M.P. 113 to 116°C); 5.68% sitosteryl propionate (M.P. 104 to 107°C); 7.87% sitosteryl palmitate (M.P. 87 to 89°C); and 9.57% "sitosteryl oleate." Again the esters, with the exception of the oleate, contained no detectable free sterol. The "sitosteryl oleate" was a mixture of free and esterified sterol with an excess of oleic and related acids. Of the sitosterol present 61% was esterified and 39% free, as determined by digitonin precipitation.

[5] Harlan Rat-Mouse Mash.

The feed for the animals receiving sitosterol or sitosterol esters was prepared in the following manner. The cholesterol dissolved in cottonseed oil was added to one-half the laboratory mash. The sitosterol or its ester was dissolved in chloroform and sprayed on the remaining half of the mash, and the chloroform removed by evaporation. The two halves were then thoroughly mixed by tumbling.

The results are summarized in table 2. Addition of 1% cholesterol to the diet again resulted in a slight increase in serum cholesterol. The further addition to the diet of 5% free sitosterol or equivalent amounts of the several esters tended to inhibit this increase. However, the differences in mean serum cholesterol levels between the various groups were small, and none differs significantly from the non-cholesterol fed controls ($P > 0.05$).

The differences between the groups are more marked with regard to liver cholesterol. Free sitosterol and the several esters all resulted in mean liver cholesterol levels lower than that of the cholesterol fed controls. ($P < 0.05$ for the palmitate and < 0.01 for the other groups.) Sitosteryl propionate and to an even greater extent plamitate were less effective than the free sterol in inhibiting cholesterol absorption. Both of these groups had significantly higher liver cholesterol levels than the group receiving free sitosterol ($P < 0.01$).

Mean liver cholesterol levels differed but little in the groups receiving free sitosterol, sitosteryl acetate and "sitosteryl oleate." To explore the possibility that 5% sitosterol was a sufficient excess to obscure differences between the free sterol and these esters, the experiment was repeated using amounts equivalent to 0.5, 1, and 2% free sitosterol. Ten groups of 4 to 6 rats of similar mean weight were employed. The 1% cholesterol and the sitosterol or its ester were added to the diet in the manner previously described.

The results are summarized in figure 1. Adminstration at these reduced concentrations failed to demonstrate any differences in the effects of the three compounds. Differences in

TABLE 2

Effect on serum and liver cholesterol of the cholesterol-fed rat of the addition to the diet of free sitosterol and various sitosterol esters

NO. RATS	ADDED TO BASIC DIET	WEIGHT		SERUM TOTAL CHOLESTEROL	LIVER TOTAL CHOLESTEROL
		Initial	Gain		
	%	*gm*	%	*mg/100 ml*	*mg/100 gm*
4	None	295	19	76 ± 6 [1]	257 ± 22 [1]
8	Cholesterol, 1	294	21	84 ± 6	1268 ± 244
4	Cholesterol, 1 + free sitosterol, 5	307	14	82 ± 13	255 ± 31
4	Cholesterol, 1 + sitosteryl acetate, 5.51	309	17	71 ± 1	300 ± 28
4	Cholesterol, 1 + sitosteryl propionate, 5.67	309	17	74 ± 6	531 ± 71
4	Cholesterol, 1 + sitosteryl palmitate, 7.87	306	16	78 ± 12	862 ± 274
4	Cholesterol, 1 + "sitosteryl oleate," 9.57	309	13	75 ± 12	211 ± 7

[1] Values given are the mean and standard deviation, estimated using (n — 1).

mean liver cholesterol at each level of administration were small and in no case statistically significant.

DISCUSSION

If the concentration of liver cholesterol is accepted as an index of cholesterol absorption in the rat it is apparent that none of the esters of cholesterol are absorbed any more

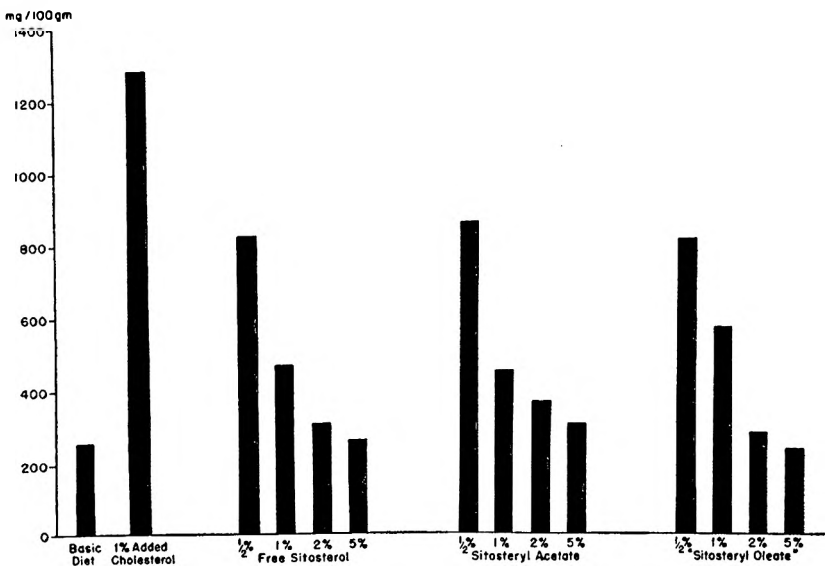

Fig. 1 Effects of the addition to the diet of the cholesterol-fed rat of free sitosterol, sitosteryl acetate and "sitosteryl oleate" in amounts equivalent to 0.5, 1, 2 and 5% free sterol. Mean liver total cholesterol of groups of 4 to 6 animals is represented.

readily than the free sterol. This result lends no support to the concept that intraluminal esterification of cholesterol serves to facilitate its absorption; were such the case one might anticipate the esters to be more readily absorbed than the free sterol.

Cholesteryl acetate and cholesteryl oleate were apparently absorbed to approximately the same extent as the free sterol. This is in contrast to the benzoate, stearate and palmitate,

which resulted in liver cholesterol only slightly higher than that of the non cholesterol fed control group.

Since the "cholesteryl oleate" was incompletely esterified it is likely that the 38% of cholesterol present in the free form is responsible in part for the apparent ready absorbability. However, the differences in the absorption of the other esters require explanation, and would seem most likely to be due to differences in their rates of hydrolysis in the intestinal tract.

Pancreatic cholesterol esterase has been shown *in vitro* to effect hydrolysis of cholesterol esters as well as the esterification of free cholesterol (Swell and Treadwell, '55). The activity of the enzyme was found to be greatly influenced by the pH and by the nature of the fatty acids. The optimal pH for hydrolysis of all the 13 esters studied was 6.6, while the optimal pH for esterification ranged from 6.1 to 4.7. The enzyme was most active in respect to hydrolysis with the short chain acids and most active in esterification with the long-chain acids. Comparison of oleic acid with stearic indicated that unsaturation facilitated both hydrolysis and esterification. From these *in vitro* studies the authors concluded that under the conditions prevailing in the intestinal lumen the enzyme would tend to produce a mixture of free cholesterol and cholesterol esters of the long-chain fatty acids, especially oleic.

Employing a partially purified cholesterol esterase derived from beef pancreas Korzenovsky [6] determined the relative rates of hydrolysis of three of the cholesterol esters employed in our study. So studied were the cholesteryl acetate, palmitate, and a purified fraction of the cholesteryl oleate. Measuring the CO_2 evolved in a CO_2-$NaHCO_3$ system the relative rates of hydrolysis were: cholesteryl palmitate 1, cholesteryl oleate 13.6, and cholesteryl acetate 32.7.

The ready absorbability of cholesteryl acetate and cholesteryl oleate may then be interpreted as a reflection of the relatively rapid rate at which they are hydrolyzed in the

[6] Personal communication.

intestine, making available free cholesterol for absorption. The much slower rates of hydrolysis of the palmitate, stearate and presumably benzoate could account for the very limited absorption of cholesterol when these esters are fed.

The results of this study of the absorbability of free cholesterol and several of its esters in the rat are in general agreement with the results obtained in chicks by Peterson, Shneour and Peek ('54). These investigators observed that free cholesterol was most readily absorbed and concluded that in the chick too the absorbability of cholesterol esters might depend upon their rates of hydrolysis.

The relative effectiveness of free sitosterol and its several esters in inhibiting cholesterol absorption would also seem to be a reflection of the amount of free sterol in the intestinal contents. Beta-sitosterol differs from cholesterol in chemical structure only in the presence of an ethyl group on carbon-24 of the side chain. As might be expected from the similarity in structure to cholesterol it has been shown by *in vitro* studies that under suitable conditions pancreatic esterase effects the hydrolysis of sitosterol esters (Swell, Field and Treadwell, '54). Korzenovsky,[7] employing the same techniques utilized in the cholesterol ester study, observed the following relative rates of hydrolysis: sitosteryl palmitate 1, sitosteryl oleate 16.2, and sitosteryl acetate 10.5.

These results indicate that, as with cholesterol esters, sitosterol esters with short-chain or unsaturated acids are more rapidly hydrolyzed than esters with the long-chain saturated acids.

The effects of free sitosterol and the readily hydrolyzed acetate and oleate at each of the various dietary concentrations employed were essentially identical. In contrast, the slowly hydrolyzed sitosteryl palmitate exhibited significantly less interference with cholesterol absorption. ($P < 0.01$).

This is in accord with the observation of Peterson et al. ('53) that esterification with capric acid largely destroyed the ability of soy sterols to interfere with cholesterol ab-

[7] See footnote 6.

sorption in the chick. Sitosteryl propionate, which might be anticipated to have an intermediate rate of hydrolysis, exhibited an effect on cholesterol absorption intermediate between that of the acetate and the palmitate. These findings are compatible with the concept that sitosterol must be present as the free sterol in order to interfere with cholesterol absorption.

These observations suggesting that it is free cholesterol which enters into the absorptive process, and that sitosterol must be in the free state to be active, lend support to the hypothesis that interference with cholesterol absorption is a result of competitive inhibition. That this interference is with a step in the absortive process prior to esterification is suggested by the previously cited studies of Daskalakis and Chaikoff ('55).

SUMMARY

Serum and liver cholesterol levels of rats were determined at the end of 14 days' maintenance on the following diets: low cholesterol, 1% cholesterol, and equivalent amounts of cholesteryl acetate, benzoate, palmitate, stearate and oleate. The liver cholesterol in the animals fed free cholesterol, cholesteryl acetate and cholesteryl oleate was significantly elevated as compared to the other groups. The cholesteryl benzoate, palmitate and stearate resulted in only minimal elevation of liver cholesterol as compared to low cholesterol diet. The increment in liver cholesterol was almost entirely in the ester fraction, without regard to whether the cholesterol added to the diet was free or esterified.

In a second study serum and liver cholesterol levels were determined at the end of 14 days' maintenance on a 1% cholesterol diet to which was added 5% sitosterol or equivalent amounts of sitosteryl acetate, propionate, palmitate and oleate. Free sitosterol and the several esters all resulted in mean liver cholesterol levels lower than that of the cholesterol-fed controls. Free sitosterol and the acetate and oleate displayed the greatest inhibitory effect on cholesterol absorption, mean liver cholesterol levels in these groups not

differing appreciably from that of the group fed a low-cholesterol diet. Sitosteryl propionate was a less effective inhibitor of cholesterol absorption, and the palmitate least effective.

The differences in absorption of the several cholesterol esters appear to be related to their relative rates of hydrolysis by pancreatic cholesterol esterase, the more rapidly hydrolyzed esters being more readily absorbed. Similarly, the effectiveness of sitosterol esters in inhibiting cholesterol absorption may be related to their relative rates of enzymatic hydrolysis, the more rapidly hydrolyzed esters being the more effective inhibitors.

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KIDNEY CHANGES IN VITAMIN E-DEFICIENT RATS

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Degeneration of the cortical tubules of the kidneys of rats deficient in vitamin E was first reported by Martin and Moore ('36, '38, '39). The abnormality was observed in animals which had been kept on their deficient diet, which contained lard as its fat component, for periods of 15 months and upwards.

More recently Emmel ('56, '57) has found that the changes reported by Martin and Moore cannot be seen in sections made from kidneys which have been fixed immediately after the death of the animal. In kidney in which fixation is delayed, however, rapid autolysis gives rise to the typical histological picture, characterized by a tendency to histolysis in the cells lining the tubules. The abnormality is nevertheless typical of avitaminosis E, since the kidneys of animals supplied with the vitamin do not show appreciable histolysis in the same time. According to Emmel, moreover, the presence in the diet of highly unsaturated fat is necessary for the production of the abnormality. In his first experiments the diet included 30% of its calories as the free fatty acids of linseed oil.

In a preliminary paper (Moore, Sharman and Symonds, '57) we have confirmed the importance of the delay in fixation, and of the influence of the dietary fat, in promoting histolysis. In our experience, however, much lower intakes of unsaturated fats sufficed for the production of post-mortem histolysis than are supplied by a high intake of the acids of linseed oil. Thus

the phenomenon could be observed when the diet contained 10% of lard. Under suitable conditions histolysis occurred in the kidneys from rats which had been deprived of the vitamin for 5 months. After more prolonged deficiency degenerative changes could be detected in the nuclei of some of the affected tubules, even when the kidneys had been fixed promptly after death. Short periods of dosing with α-tocopherol, commenced after periods of deficiency sufficiently long to cause histolysis, were ineffective in its prevention. In such animals, however, the histolysis could be prevented by prolonged dosing. Methylene blue, which can replace vitamin E in at least some of its functions (Dam, Kruse, Prange and Sondergaard, '51; Moore, Sharman and Ward, '53) was found effective in preventing renal histolysis.

The purpose of this paper is to describe the foregoing work more fully, and also to report further experiments on the same problem.

MATERIALS AND METHODS

Rats. Most of our rats were pure-bred piebalds, but in some experiments pure-bred albinos were used. Both sexes were employed. The animals were given their experimental diets from weaning.

Diets. Our standard diet for inducing vitamin E deficiency in rats consists of: casein (vitamin-free) 25, sucrose 50, lard 10, dried brewers' yeast 10 and minerals, 5%. Vitamin A was usually supplied as 1000 I.U. of synthetic vitamin A acetate weekly, vitamin D as 60 I.U. (1.5 μg) of ergocalciferol and vitamin K as 50 μg of menadione, each dose being dissolved in one drop (20 mg) of arachis oil. In some experiments, for convenience only, one drop of halibut liver oil replaced the solutions of vitamins A and D. When required, vitamin E was administered as a solution of *dl*-α-tocopheryl acetate, in a 5% solution in arachis oil.

Experimental modifications of the standard diet were made by varying the amount of lard, or by omitting the lard in favor of cod-liver oil, linseed oil, coconut oil, or extra

sucrose. When the fat component of the diet was varied from the usual 10% an adjustment was made, on a weight for weight basis, in the percentage of sucrose. Another modification was the omission of yeast and its replacement by a mixture of water-soluble vitamins, to give a diet as follows: casein (vitamin free) 30, sucrose 65 and minerals 5%; plus choline chloride 200 mg, Ca-*d*-pantothenate 2 mg, nicotinamide 2.5 mg, thiamine 300 μg, pyridoxin 300 μg, riboflavin 300 μg and biotin 10 μg. Vitamins A, D, K were added as weekly supplements.

Dietary fats. The various fats were examined for unsaturation by a modification of the method of Wijs, and for vitamin E by a method involving two dimensional chromatography (Green, Marcinkiewicz and Watt, '55). The results were as follows:

	I.V.	TOCOPHEROL *mg/100 gm*
Lard	50	0
Cod-liver oil	154	10.2 (all α)
Linseed oil	181	20 (all γ)
Coconut oil	11	0

Experimental procedure. The animals were kept on their diets, some with weekly doses of α-tocopherol, for periods of from three to 15 months. In some experiments dosing with tocopherol was delayed until after a period of avitaminosis had been imposed. After the desired dietary preparation the animals were killed by coal gas. For use as a control, without autolysis, one kidney was usually dissected out at once and fixed promptly in formol saline. In order to allow adequate fixation the kidney was bisected longitudinally, with a central cut made from the cortex and through the papilla. The other kidney was left *in situ* in the carcass of the animal, covered by the remaining viscera, for exactly three hours, during which time the carcass was kept at room temperature (about 22°C). The second kidney was then removed and was fixed in the same way as the first. Paraffin sections (5 μ) were made as a routine, and were stained with hematoxylin and eosin. For selected animals, however, different methods of staining

were applied to paraffin sections, or frozen sections were prepared.

Grading of histolysis. In order to compare the influence of the various dietary treatments in inducing histolysis a method for grading its intensity was required. As a simple expedient three sections were selected, from the large collection available, as typifying mild (Grade 1) medium (Grade 2) and advanced (Grade 3) histolysis. Photomicrographs of these sections, and also a control section from a rat dosed prophylactically with α-tocopherol, are shown in figure 1.

Parallel studies on other abnormalities. All the animals which were used for the study of kidney abnormalities were also examined for various well-known indications of vitamin E deficiency, including degeneration of the testes, brown uterus and an increased liability to hemolysis, *in vitro,* by dialuric acid. The results of these examinations need not be reported in full in the present communication, but they will be mentioned in the discussion when they can help in the interpretation of our findings for the kidneys. Our rats were also inspected for scaliness of the tail, as an indication of deficiency of essential fatty acids.

OBSERVATIONS

Renal histolysis in relation to vitamin E and fat. The results of two series of experiments are summarized in table 1. In the first series most of the rats received their diet for about 5 months. In the second the period of feeding was extended to 6 or 8 months. The kidneys, removed three hours after death from each rat, were examined microscopically and were graded for histolysis, as indicated by the histological picture. In each group the variation was usually slight, and only the average grading is given in table 1. Sex had no obvious influence on the liability to histolysis.

It will be seen that kidney histolysis was invariably absent in all the 5 groups (2, 11, 16, 18 and 20) which were dosed with α-tocopherol. Thus the vitamin, in an adequate dose of 2 mg of its acetate weekly, gave protection irrespective of

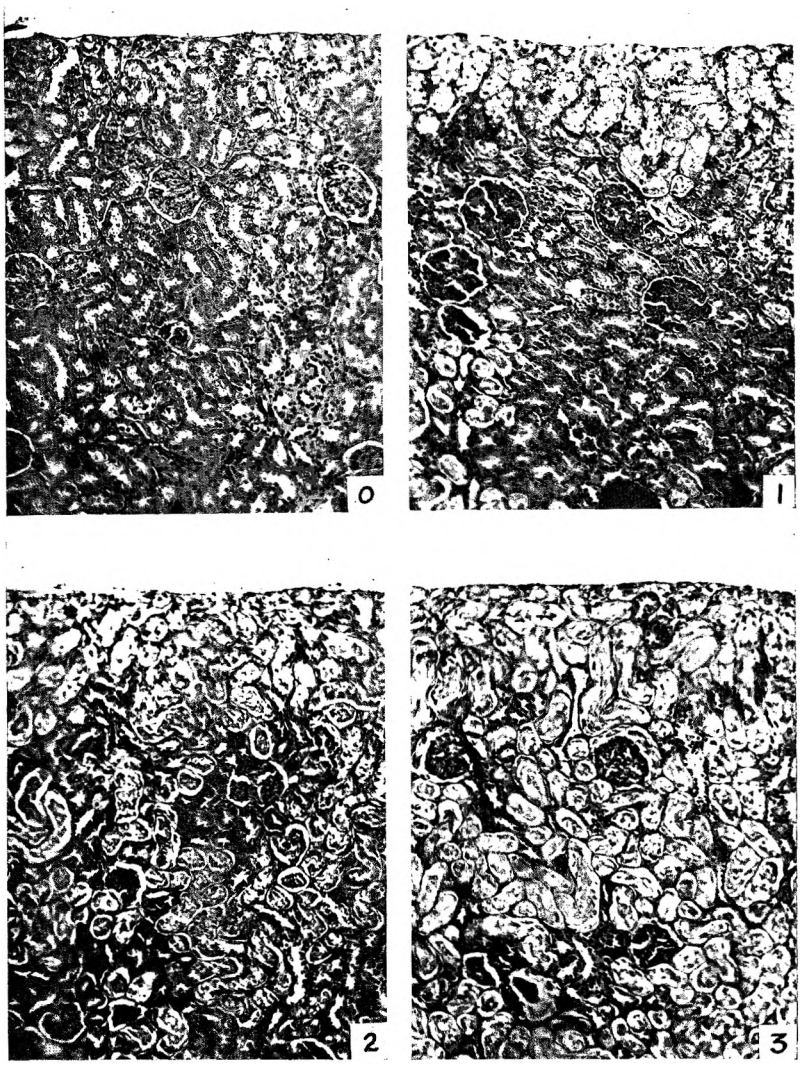

Fig. 1 Renal histolysis markings. All the kidneys were from female rats, and had been fixed exactly three hours after the death of the animal. Paraffin sections were cut, and stained with haematoxylin and eosin. Photomicrographs of the cortical tubules were taken at low magnification. *Grade 0.* No appreciable histolysis. The kidney was taken from a rat which had received a basal diet deficient in vitamin E for 145 days, but with supplements of 2 mg of *dl*-α-tocopheryl acetate weekly. *Grade 1.* Slight histolysis. The kidney was taken from a rat which had been deprived of vitamin E for 174 days. *Grade 2.* Extensive histolysis. Rat deprived of vitamin E for 510 days. *Grade 3.* Almost complete histolysis. Rat deprived of vitamin E for 467 days. Sections which appeared to fall between these grades were marked 0.5, 1.5 or 2.5.

the nature of the dietary fat. In the lard groups the extent of histolysis increased with the experimental period, and with the percentage of the lard in the diet. Cod-liver oil, 10 or 30%, caused almost complete histolysis in the rats given their diet for 144 days. After 210 days some degree of

TABLE 1

Post-mortem renal histolysis in rats given diets containing various fats, with and without supplements of vitamin E

GROUP NO.	NO. OF RATS ♂	NO. OF RATS ♀	DIETARY FAT	α-TOCOPHEROL	AVERAGE DAYS ON DIET	AVERAGE RENAL HISTOLYSIS GRADING [1]
			%			
1	5	3	Lard, 10	—	144	0.63
2	3	2	Lard, 10	+	144	0
3	3	3	Lard, 30	—	144	1.9
4	3	3	No fat	—	145	0
5	3	3	No fat, no yeast	—	144	0
6	3	3	Cod-liver oil, 10	—	144	2.7
7	3	3	Cod-liver oil, 30	—	144	2.8
8	3	3	Lard, 2	—	229	0.5
9	3	2	Lard, 5	—	226	1.4
10	3	3	Lard, 10	—	223	2.0
11	4	3	Lard, 10	+	226	0
12	3	1	No fat	—	216	0
13	3	2	Cod-liver oil, 2	—	210	1.0
14	2	1	Cod-liver oil, 5	—	210	2.8
15	3	2	Cod-liver oil, 10	—	219	2.8
16	3	3	Cod-liver oil, 10	+	221	0
17	3	3	Linseed oil, 10	—	222	0.3
18	3	3	Linseed oil, 10	+	222	0
19	3	3	Coconut oil, 10	—	222	0
20	2	3	Coconut oil, 10	+	222	0

[1] Grade 1, mild; grade 2, medium, and grade 3, advanced histolysis.

histolysis was caused by only 2% of cod-liver oil, while with 5% of the oil histolysis was virtually complete. Linseed oil, in spite of its high iodine value, caused only partial histolysis in one out of 6 rats. There was no histolysis when the diet contained coconut oil.

Minimal dosage of α-tocopherol for preventing renal histolysis. In our experience a weekly dose of 0.25 mg of *dl*-α-

tocopheryl acetate has usually given complete, or almost complete, protection against brown uterus, or degeneration of the testes, in rats given our standard diet containing 10% of lard. It seemed important to find out, for reasons which appear later, whether the same level of dosing could protect against kidney histolysis. An experiment was set up, therefore, in which female rats were fed upon our standard diet for 158 to 167 days, and were given weekly doses of 0, 0.25,

TABLE 2

Influence of graded doses of vitamin E on post-mortem renal histolysis

GROUP NO.	WEEKLY DOSE OF α-TOCOPHEROL ACETATE	NO. OF RAT IN GROUP	DAYS ON DIET	BROWN UTERUS, MARKING	RENAL HISTOLYSIS MARKING [1]
	mg				
21	0	1	158	5	2
		2	167	3½	2
		3	167	3	2
22	0.25	1	167	1	0
		2	167	1½	0
		3	167	1½	0
23	0.5	1	167	½	0
		2	167	0	0
		3	167	0	0
24	0.75	1	167	0	0
		2	167	0	0
		3	167	0	0

[1] See footnote to table 1.

0.5 or 0.75 mg of *dl*-α-tocopherol. The animals were then killed for inspection of the uterus, and for examination of histolysis in the kidneys three hours after death. The results are given in table 2.

It will be seen that the lowest dose of 0.25 mg weekly gave complete protection against renal histolysis, when studied according to our standard technique. The same level of dosing gave partial, but not complete protection against brown uterus.

The prevention of renal histolysis by prolonged curative dosing with tocopherol. In order to study the response of the kidneys to curative treatment female rats were first kept on our standard vitamin E-deficient diet for periods long enough to ensure a liability to renal histolysis in all the animals. Some were then given single or repeated doses of α-tocopherol, while others were killed after remaining on the basal diet, but without supplements, to make up the same total periods. The results obtained are given in table 3.

TABLE 3

The prevention of post-mortem renal histolysis by curative dosing with vitamin E

GROUP NO.	NO. OF RAT IN GROUP	PERIOD OF DEPRIVATION	DOSE OF α-TOCOPHEROL	PERIOD OF DOSING	TOTAL EXPERIMENT PERIOD	RENAL HISTOLYSIS GRADING [1]
		days	*mg*	*days*	*days*	
25	1	268	0	0	268	2
	2	267	2 (single)	1	268	2
	3	267	2 (single)	1	268	2
	4	235	2/week	33	268	2
	5	338	0	0	338	3
	6	338	0	0	338	2
	7	267	2/week	71	338	2
	8	267	2/week	71	338	1
	9	235	2/week	103	338	0.5
	10	235	2/week	103	338	0

[1] See footnote to table 1.

It will be seen that a single dose of 2 mg of α-tocopherol was ineffective in preventing histolysis, when the rats were killed one day later. When the same dose was given weekly for about 5 weeks it was also ineffective. In two animals which were dosed for 10 weeks the tendency to histolysis appeared to be reduced in one, but not in the other. In two animals dosed for 15 weeks there was no histolysis in one, and only slight autolysis in the other.

The prevention of renal histolysis by methylene blue. In a first experiment the basal diet contained lard. A control group was given no supplements, but two other groups received α-tocopherol, as weekly doses of 2 mg of the acetate, or methylene blue, as 0.126% of their diet. In another experiment the diet contained cod-liver oil, with or without the same supplements. The results are summarised in table 4.

It will be seen that methylene blue completely prevented renal histolysis, either in rats kept for about 7 months on the diet containing lard or in others kept for 2½ months on the diet containing cod-liver oil.

TABLE 4

The prevention of post-mortem renal histolysis by methylene blue

GROUP NO.	NO. OF RATS	DIETARY FAT	α-TOCOPHEROL GIVEN, WEEKLY	METHYLENE BLUE GIVEN	AVERAGE DAYS ON DIET	AVERAGE RENAL HISTOLYSIS GRADING [1]
		%	*mg*	%		
26	3	Lard, 10	—	—	203	2.2
27	3	Lard, 10	2	—	203	0
28	4	Lard, 10	—	0.126	203	0
29	3	Cod-liver oil, 10	—	—	75	3.0
30	3	Cod-liver oil, 10	2	—	75	0
31	3	Cod-liver oil, 10	—	0.126	75	0

[1] See footnote to table 1.

Renal abnormalities not attributable to autolysis. In addition to the observations which have already been described and which were all made on kidneys fixed three hours after death, examinations were usually made on kidneys which had been fixed promptly after the death of the animal.

In the tubules of promptly fixed kidneys taken from rats which had received our standard lard diet, for periods barely adequate to cause histolysis after delayed fixation, no abnormality was detected in routine examination. In kidneys from rats which had received the diet for 7 months or more, however, changes were often noticed in some of the tubules. Thus a few of the nuclei were no longer stained by hematoxylin, but had a golden brown color. The abnormalities could

be demonstrated clearly by staining frozen sections with hematoxylin and Sudan 4. With this treatment some cross sections of tubules presented a normal purple color, but others appeared brown, presumably because of the presence of abnormal lipids. In rats which had received cod-liver oil much the same abnormalities were seen as resulted from the lard diet, but their development tended to be more rapid.

The abnormal tissues were strongly stained by eosin, neutral red, methyl violet and malachite green, which left normal tissues only faintly colored. Figure 2A shows a paraffin section, stained with neutral red, from a rat which had received our lard diet, without tocopherol supplements, for about 17 months. In some of the tubules masses of darkly staining material, globular in shape, have either replaced the normal nuclei, or have adopted positions in near proximity. In other tubules the darkly staining material appears as clusters of small granules.

Figure 2B is another paraffin section, also stained with neutral red, from a rat which had received for 8 months a diet containing 10% of cod-liver oil, again without tocopherol. Normal cells, and others in various stages of abnormality may be seen. In normal cells the nuclei stain lightly and evenly, except for their darker nucleoli and chromatin granules. They are differentiated only slightly from the surrounding cytoplasm. Other cells appear normal in size and position, but a deeply staining mass has appeared in their center, which is separated by a clear space from a surrounding membrane. Their appearance suggests, at first sight, that abnormal nuclear inclusions have condensed at the center of the nucleus. An alternative interpretation, however, might be that the nuclear membrane has disappeared, and also the staining parts of the cytoplasm, leaving the abnormal nucleus at the center of a distended cell membrane. Finally some cells, with a mass of deeply staining material at their center, have become so enlarged that they protrude into the lumen of the tubules. They appear to be on the point

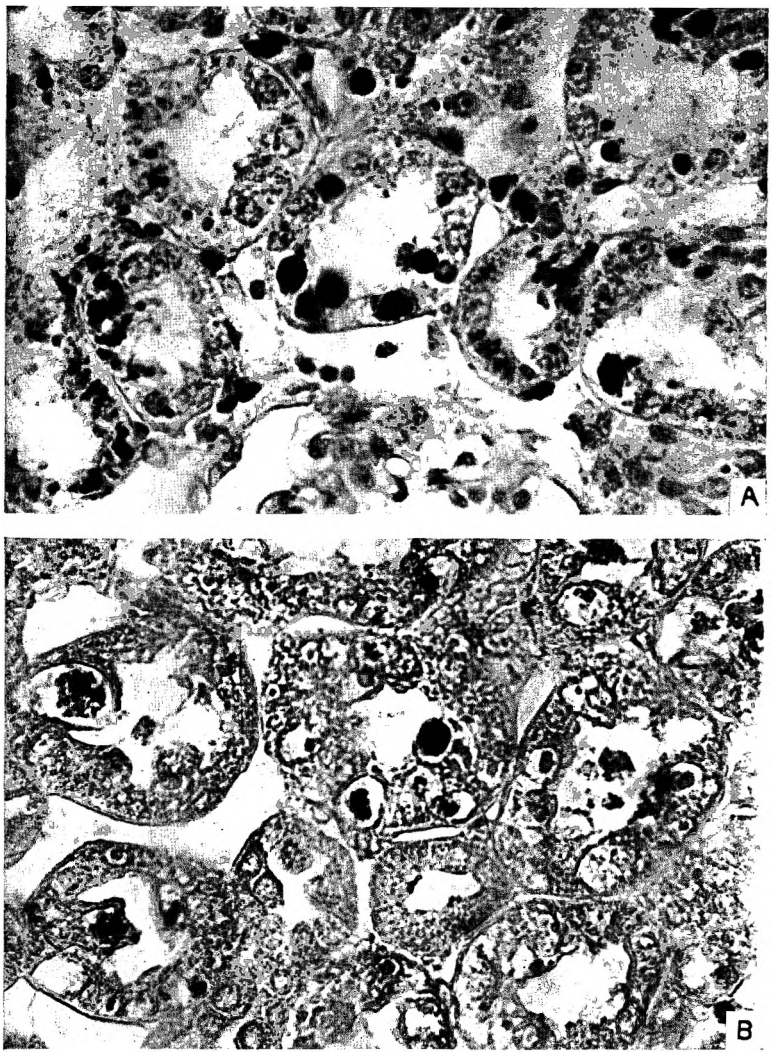

Fig. 2 Kidney abnormalities not involving autolysis. The kidneys were fixed as soon as possible after the death of the animal. Paraffin sections of the cortical tubules were cut, and stained with neutral red. Photomicrographs were taken under high magnifications. A, Kidney from a female rat which had received a diet containing 10% of lard, without supplements of tocopherol, for 520 days. Note the accumulation of deeply staining material in some of the tubules. B, Kidney from a male rat which had received a diet containing 10% of cod-liver oil for 231 days, without supplements of tocopherol. Note again the accumulations of deeply staining material.

of bursting, which would account for the frequent appearance of deeply staining material in the lumen.

The changes in promptly fixed kidneys were studied less extensively than those after autolysis, and these findings must be regarded as preliminary indications. A point requiring further investigation was the presence of some abnormal nuclei in the kidneys of some of the animals which received a diet containing cod-liver oil, but with supplements of tocopherol.

DISCUSSION

Our results confirm Emmel's conclusion that the degeneration of the renal cortical tubules of rats deficient in vitamin E, as seen in the histological picture first reported by Martin and Moore, requires the intervention of autolysis for its development. We have also confirmed his finding that besides avitaminosis E and autolysis a third factor, the presence of unsaturated fatty acids in the diet, is necessary for the production of the typical extensive histolysis.

In regard to the nature and quantity of the unsaturated fatty acids which promote histolysis our findings diverge from those of Emmel. In his communication the necessity of including highly unsaturated fat in the diet was emphasized, and, in order to promote autolysis, his rats were given large amounts of linoleic and linolenic acids. We have found that such drastic steps are unnecessary. Histolysis can be avoided, it is true, by cutting down the proportion of unsaturated fat in the diet to a point when there is danger of deficiency of essential fatty acids. Scaly tails, an indication of this deficiency, were in fact observed in our rats of groups 4 and 5, in which renal histolysis was prevented by the complete omission of the fat component from the diet. Histolysis was also prevented, but without causing scaly tails, when coconut oil (I.V. 11) was the dietary fat. In the other direction, however, renal histolysis occurred when the diet contained as little as 5% of lard. This fat has about the same degree of unsaturation as that usually found in the rat's own body fat.

Cod-liver oil, (I.V. 154) when included in the diet, greatly intensified the tendency to renal histolysis. Our experience with linseed oil (I.V. 181), however, was surprising. In spite of its high degree of unsaturation, this fat was much less active in promoting histolysis than lard, of I.V. 50. No explanation could be found in the tocopherol contents of the various fats. Thus the 10.2 mg of α-tocopherol per 100 gm found in cod-liver oil exceeded in biological value the 20 mg of γ-tocopherol found in linseed oil, assuming that γ-tocopherol has only 20% of the potency of the α form. As a working hypothesis we may infer that various polyethylenic acids differ in their powers of promoting histolysis. An investigation of the histolytic power of arachidonic acid might be rewarding.

The dual potency of cod-liver oil, in supplying both vitamin E and fatty acids which oppose the action of this vitamin, has already been discussed by Moore, Sharman and Ward ('58). In the present investigation groups of rats which were given the diet containing 10% of cod-liver oil must have received at least 0.7 mg of α-tocopherol weekly. This was about three times the dose, not necessarily the minimum, which we found to be effective in preventing renal histolysis when the diet contained lard (table 2). When the diet contained 30% of cod-liver oil about 9 times the dose effective on a lard diet must have been given, but still renal histolysis was not prevented. In the prevention of histolysis, therefore, it is not necessary merely that vitamin E should be present in the diet. Its amount must be regulated in accordance with the strain imposed by the unsaturated fatty acids which are also contained in the diet. Moreover we have found (Moore, Sharman and Ward, '58) that the combined effects of the vitamin and its antagonists, as supplied by cod-liver oil, are different according to the tissue under investigation. Thus the inclusion of 10% of cod-liver oil in the diet protects against brown discoloration of the uterus and degeneration of the testes, at least for several months. On the other hand, there is no protection against brown discoloration of the body fat

or hemolysis by dialuric acid, while we have seen that the tendency to renal histolysis is intensified.

In regard to curative dosing with α-tocopherol, our results agree with those of Emmel, who has found that the liability to renal histolysis, once incurred, is not readily eliminated. By prolonged dosing, however, we have restored normal resistance to histolysis in a few animals. It appears that it is not merely sufficient to restore vitamin E to the kidney cells; they must be given time to react slowly to its presence. We are also in agreement with Emmel's statement that he has found methylene blue effective in preventing renal histolysis. In his work the basal diet contained the fatty acids of linseed oil, whereas in our experiments the dye was effective with either lard or cod-liver oil as the dietary fat.

Our preliminary findings on the histological changes which can be observed, without allowing autolysis, in the kidneys of rats submitted to prolonged avitaminosis E recall an early report by Mason and Emmel ('45). These workers, like ourselves, noticed the presence of globules of brown pigment in the epithelium of the proximal tubules of such animals, but did not discriminate between abnormalities which were, or were not, due to autolysis. An interesting possibility arises that a specific site for the antagonism between vitamin E and polyethylenic acids may be found in or near the nuclei of the tubules, where the sequence of events in this antagonism might be studied. Obviously the possible identity of the abnormal material with the well known ceroid (Lillie, Ashburn, Sebrell, Daft and Lowry, '42) requires investigation. We must emphasize, however, that whereas the changes seen in promptly fixed tissues affect only a minority of the tubular cells, those changes which are seen after autolysis can lead to the disintegration of every tubular cell in the whole cortex. An abnormal condition of the nucleus, as observed after prompt fixation, is therefore not a prerequisite for histolysis. The possibility remains, of course, that the liability to histolysis and the gross nuclear abnormalities are early and late stages in the same pathological process.

SUMMARY

1. Rats were kept on diets deficient in vitamin E, with or without supplements of tocopherol and containing various fats, for prolonged periods. They were then killed, and histological sections were made from their kidneys. One kidney from each animal was fixed promptly in formol saline, but the other was allowed to remain in the carcass for a standard period before fixation.

2. By comparisons of sections from kidneys in which fixation had been prompt or delayed the role of autolysis in producing the typical histolysis in the cortical tubules, indicative of vitamin E deficiency, was confirmed.

3. The intensity of post-mortem renal histolysis was influenced by the nature and quantity of the dietary fat. Histolysis regularly occurred with a standard vitamin E-deficient diet containing lard. With cod-liver oil in the diet histolysis was intensified. No histolysis occurred, however, when the fat was coconut oil, or when additional carbohydrate replaced the fat component. Linseed oil, in spite of its high degree of unsaturation, had very little potency in promoting histolysis.

4. Post-mortem renal histolysis was prevented by the administration of adequate weekly supplements of *dl*-α-tocopheryl acetate, irrespective of the nature of the dietary fat. The addition of methylene blue to the diet also prevented histolysis.

5. The tendency to post-mortem renal histolysis, once incurred, was difficult to correct, and responded only to prolonged dosing with α-tocopherol.

6. After rats had been restricted for very long periods to a vitamin E-deficient diet containing lard, or for somewhat shorter period to a diet containing cod-liver oil, renal abnormalities could be observed without the intervention of autolysis. Thus changes were apparent in some of the nuclei of the cortical tubules even when the kidney had been fixed promptly after the death of the animal.

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BEHAVIORAL CHANGES IN RATS AND GUINEA PIGS INDUCED BY THE ADMINISTRATION OF INDOLE 3-ACETIC ACID AND 6-AMINONICOTINAMIDE

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The plant-growth hormone indole 3-acetic acid (I3–AA) or heteroauxin is structurally similar to tryptophan and serotonin. Although it has been suggested as an inhibitor of the synthesis of niacin from tryptophan by Kodicek, Carpenter and Harris ('46) it is not regarded as a "pellagragenic" substance according to Rosen and Perlzweig ('47). In human patients Jolliffe, Bowman, Rosenblum and Fein ('40), and Sydenstricker and Cleckley ('41) found that niacin deficiency may produce encephalopathy in the absence of characteristic dermatologic or gastrointestinal symptomology.

In contrast to I3–AA, 6-aminonicotinamide (6–AN) is a potent niacin antagonist as shown by the work of Johnson and McCall ('55). Experiments performed by Efremov, Maxarychev and Tikhomirova ('55) with dogs on a restricted niacin intake showed a disturbance of conditioned reflexes which appeared before the usual clinical symptoms of deficiency were apparent. In view of these findings, an investigation of the behavioral effects of I3–AA and 6–AN was undertaken on rats and guinea pigs.

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EXPERIMENTAL

A total of 68 rats was used, the animals being divided into three separate groups of 20 to 24 each. The study was conducted over a period of 8 months. Originally of the Sprague-Dawley strain, all rats were third generation pen inbreds. Each animal was caged individually. A preparatory period of two to three weeks preceded the administration of I3–AA to experimental animals or placebo to controls. During this time the animals became accustomed to the investigators, attaining a stable level of tractability.

Rats of both sexes were used, the average age being 200 days. The average weight of the males was 320 gm and of the females 235 gm. The colony was maintained on a commercial laboratory chow [3] to which the animals were accustomed. Food intake was ad libitum for controls and experimentals.

The rats were given I3–AA orally in daily doses. The initial dosage of 0.1 ml, containing 1 mg in an ethanol solution, was placed on a 1 cm^3 cube of white bread weighing approximately 200 mg. Control animals received a similar volume of ethanol on a bread cube. The preparation was allowed to stand half an hour before feeding and at the end of this time there was no odor of alcohol.

A program for evaluating behavior was devised in which the animals were weighed and handled every two days and the impressions of the investigators were noted. In addition, at weekly intervals the rats were examined in detail for changes in posture, within the cage and outside of it; activity inside the cage and upon handling; response to an external stimulus in which a small pointed object (a pencil) was placed in the cage in the immediate vicinity of the animal; and in the appearance of the face and tail for evidence of dermatitis or alopecia and for indications of normal grooming. These observations were made in the evening at the period of peak activity.

[3] Ralston Purina Company, St. Louis, Missouri.

An effort was made to reverse the effects of I3–AA in several rats at the end of 12 weeks by the administration of niacin, niacinamide and tryptophan while still giving I3–AA. Reversal was also tried with the entire third group in which the experimental animals were placed on a control regimen at the end of 10 weeks.

The results achieved with rats were of sufficient interest so that two additional experiments were planned with 32 guinea pigs, ranging in age from 6 to 12 months. The guinea pigs refused oral ingestion of I3–AA so subcutaneous injections of a dilute ethanol-physiological saline solution were used. The dosage of 4 mg/kg of body weight was approximately the same as that used with the rats. Control guinea pigs were given injections of saline containing a comparable amount of alcohol.

Tests of the righting reflex of the guinea pigs were made by holding each animal supine in its cage until it had ceased struggling, generally 1 to 5 seconds, when it was released. The time before the animal regained its feet was noted. The first tests were run at the end of the 4th week of injections on two successive days. The possibility of a training effect was considered so no further tests were run until the end of the 9th week.

When any experimental guinea pigs remained supine for more than 30 seconds after being released, tests were made in terms of their response to stimuli. Their paws were struck gently, abdominal hairs were pulled, their nostrils and ears were tickled and investigators clapped their hands above them.

A further test of the righting reflex was made by cradling the animals on their backs in an investigator's hands, 18 inches above a sponge rubber pad. They were dropped and their posture upon landing was noted.

In the experiments with 6–AN, 8 guinea pigs were used. Subcutaneous injections of 1 mg were given daily to 4 animals, two of which also received 5-mg injections of niacinamide. The remaining 4 guinea pigs received 2 mg of 6–AN, two of these also receiving 10-mg injections of niacinamide.

RESULTS

At the end of one week on a dosage of 1 mg I3–AA, there was a noticeable lessening of the tractability of several of the male rats in the first experimental group. A progressive

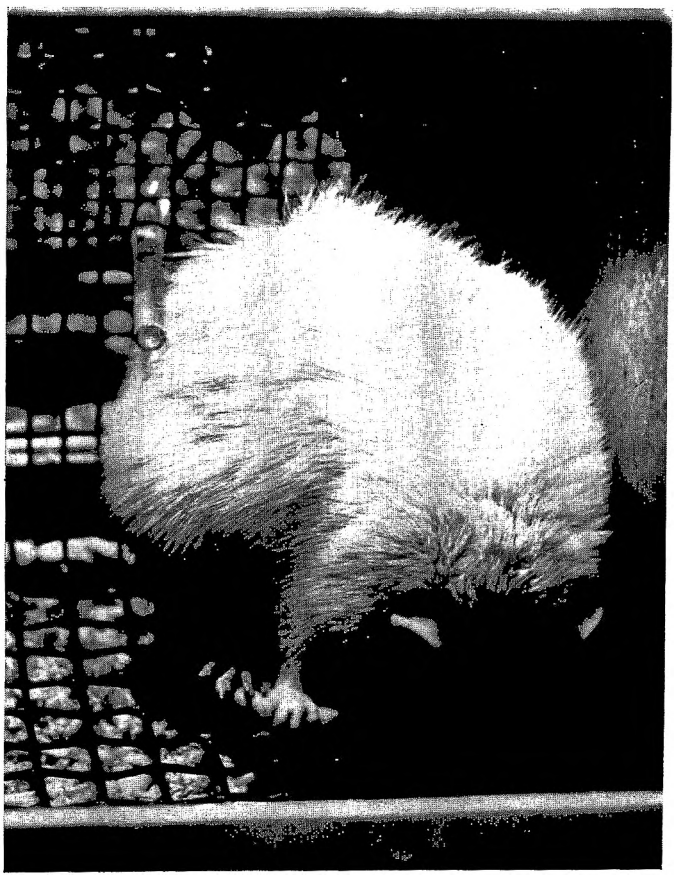

Fig. 1 The typical hunched posture of the affected rats.

change in what had been stable behavior continued until at the end of 5 weeks, a definite pattern of alteration was found. This is shown in figure 1 and was manifested to a greater or lesser degree by more than half of the experimental males. The changes in posture showed the animal hunched, in an

attitude similar to that of sleep but with eyes open. Total activity was reduced. Response to stimuli was diminished, the animal failed to show normal curiosity, and stayed in a rear corner of the cage. The coat was greasy and matted and the tail soiled. The apparent physical condition of all animals was nontheless good as far as could be told from the weight curves. These curves showed the controls, unaffected experimentals and affected experimentals to be increasing in weight at the usual rate for rats of their size and age. The number affected and the degree of change are shown in table 1 under group 1. Behavioral changes were not observed in controls.

The term "marked change" was reserved for those animals which displayed the full spectrum of behavioral abnormalities mentioned. A "moderate change" was one in which activity and response were less profoundly modified but still showed a decided difference from the controls. An evaluation of no change indicates that no behavioral alteration was shown and that these rats were indistinguishable from the controls. The control rats remained completely tractable and normally active throughout the study.

Following the examination the dosages were increased in 1-mg increments among the unaffected experimental animals until a behavioral alteration was noted or definitely failed to appear by the 12th week. This stepwise increase was continued to a level of 5 mg of I3–AA per day. The results of the final examination at the end of the 12th week are shown in table 2 under group 1. The succeeding two groups were subjected to treatment identical with that of group 1 and the data from these groups are in tables 1 and 2 under groups 2 and 3. The controls remained unchanged in behavior. The dosages were increased to 10 mg of I3–AA/day among the unaffected experimentals in group 3 for one week but no change was noted.

Peculiarities among the affected experimental rats in addition to those already described deserve notice. When an investigator would attempt to remove any of the markedly affected animals from their cages for weighing, they would

TABLE 1

Degree of behavior change in rats receiving 1 mg/day indole 3-acetic acid: first 5 weeks

BEHAVIOR CHANGE	GROUP 1				GROUP 2				GROUP 3	
	Experimental		Control		Experimental		Control		Experimental	Control
	8 ♂	8 ♀	4 ♂	4 ♀	12 ♂	4 ♀	6 ♂	2 ♀	14 ♂	6 ♂
Marked	5	1	0	0	5	0	0	0	6	0
Moderate	0	0	0	0	0	0	0	0	2	0
None	3	7	4	4	7	4	6	2	6	6

TABLE 2

Degree of behavioral change in rats receiving indole 3-acetic acid: sixth through twelfth weeks

(Effective individual dosage levels noted within parentheses)

BEHAVIOR CHANGE	GROUP 1				GROUP 2				GROUP 3	
	Experimental		Control		Experimental		Control		Experimental	Control
	8 ♂	8 ♀	4 ♂	4 ♀	12 ♂	4 ♀	6 ♂	2 ♀	14 ♂	6 ♂
Marked	5 (1 mg)	1 (1 mg)	0	0	5 (1 mg) 1 (3 mg) 1 (4 mg)	1 (1 mg) 1 (3 mg)	0	0	6 (1 mg)	0
Moderate	1 (4 mg) 1 (5 mg)	4 (5 mg)	0	0	2 (3 mg)	1 (4 mg)	0	0	3 (3 mg) 1 (4 mg)	0
None	0	2 (5 mg)	4	4	3 (5 mg)	1 (5 mg)	6	2	4 (5 mg)	6

cling to the cage bottom, squeal, and when dislodged, fight the handler. When placed upon the weighing pan however, they would remain motionless. The unaffected experimentals and the controls were always eager to leave the cage and to be handled. The affected animals were entirely unresponsive to the introduction of a pointed object (pencil) to their cages, even when prodded or touched on the nose or in the eye.

The affected experimental rats, even when totally quiescent in a cage corner displayed an excessive degree of muscular tonus with tight muscles when touched or lifted. The pattern of droppings and food crumbs in the cage pans indicated that little time was spent away from the preferred rear corner of the cage. One rat was noted frequently over a period of two days to be standing with his nose pointed toward an upper rear corner of his cage. He would leave this position to secure and eat a food pellet, then return to it immediately.

At the end of the 10- or 12-week periods the experimental rats exhibited neither skin lesions, alopecia nor diarrhea and the incidence of respiratory distress was about the same as in the control group.

Reversal was attempted in two ways. The administration of 10 mg/day of niacin, 10 mg/day of niacinamide or 10 mg/day of tryptophan, while still giving I3–AA, showed no clear benefit. In the third group however, when I3–AA was discontinued for 5 weeks after 10 weeks of administration, 5 of the 6 rats showing a marked behavior change were not benefited. Of 5 rats showing a moderate degree of behavior change, three were reevaluated as normal.

In the first group of 14 guinea pigs there was no notable change in gross behavior at the end of 4 weeks but a test of the righting reflex showed a marked difference between controls and experimentals. The 5 control guinea pigs took a mean of less than 1 second (0 to 3) to regain a right posture. The 9 experimental animals' mean righting time was 158 seconds (5 to 585). The sex difference shown by the rats in tables 1 and 2 was not apparent in guinea pigs. The mean of the 4 experimental females was 167 seconds (7 to 575), and

of the 5 males experimental 148 seconds (5 to 253). The immediately apparent difference between the control and experimental groups prompted a repeat of the test on the following day. These results were; controls, mean of 5 seconds (0 to 16), experimentals, mean of 144 seconds (19 to 269). The mean of the experimental females was 130 seconds (22 to 250) and of the males, 156 seconds (19 to 269).

No further tests were run until the end of the 9th week when the control mean was found to be 20 seconds (3 to 30) and that of the experimentals to be 209 (22 to 461). The mean of the experimental females was 238 (22 to 378) and of the males was 187 seconds (47 to 461).

Since the righting reflex time prior to the administration of I3–AA was not known, a second experiment was performed with 18 new guinea pigs. Their righting reflex times before receiving I3–AA were uniformly zero to three seconds. The animals were divided into experimental and control groups, on the basis of equality of size, age and righting time.

At 4 weeks this experiment showed the 7 control animals to have a mean righting time of 1.4 seconds (0 to 5) and the 11 experimentals to have a mean of 117 (17 to 203). At 10 weeks the mean of the controls was 19 seconds (14 to 30) and that of the experimentals was 148 seconds (16 to 360). The figure for experimentals was for 10 animals only. The remaining guinea pig's time of 1036 seconds (17 minutes, 16 seconds) was not included in the group mean.

When tests were made of the response to stimuli of the guinea pigs showing loss of the righting reflex, it was found that leg movements followed striking of the limb, nose and ear twitching always followed tickling and the animals often squealed after hair pulling or hand clapping, but at no time did the animals thus treated respond by regaining a righted posture.

The dropping test showed that the experimental animals never regained a righted posture while falling although the controls always landed on their feet. When the distance of

the drop was raised to 24 inches, the experimentals still fell on their backs or sides.

The experiment using 6–AN showed that the quinea pigs which were given the niacin antagonist alone evidenced a weight loss beginning on the second day with diarrhea appearing on the third day, resulting in death on the 5th day for the animals receiving 2 mg of 6–AN. The two guinea pigs receiving 1 mg of 6–AN per day died on the 6th and 7th days.

When protective doses of niacinamide were given to the 4 other guinea pigs there was no weight loss or diarrhea. At the end of 4 weeks on the dual injections there was no noticeable impairment of physical condition. When these animals were tested for righting reflex times at the end of three weeks it was found that none righted in less than 30 seconds, with a mean time of 73 seconds. Retesting at the end of the 4th week showed an increase to 94 seconds (45 to 148).

DISCUSSION

The data indicate that in a reasonably homogeneous rat colony with a known, stable behavior pattern I3–AA can produce a change in this pattern in 19 of 46 animals in 5 weeks and in 36 of 46 animals in 10 to 12 weeks. The initial susceptibility of males is greater than that of females. The dosages used were relatively small suggesting a sensitive site of action. The range of effectiveness and variations in individual susceptibility indicate that a competitive situation may be involved, and that I3–AA may be a metabolic antagonist acting against a receptor with some reserve capacity. The apparent absence of somatic symptomology also indicates that a highly susceptible system was affected.

The effect of I3–AA upon the righting reflex of guinea pigs shows that in this species, at least, there may be a functional involvement. The lower susceptibility of the female rat to behavior change was not borne out in the result with guinea pigs, in which the females seemed to be as sensitive as the males.

The guinea pigs receiving 6–AN exhibited a picture of the action of an antimetabolite against niacinamide. When, however, niacinamide was given to protect against weight loss, diarrhea and death, the somatic effects failed to appear but the delay in the righting reflex, though mitigated, was still retained.

The effects of I3–AA on guinea pigs were similar to those observed in guinea pigs protected by niacinamide from 6–AN, namely that only the prolonged time for the initiation or righting was observed. In rats, however, even when unprotected by the additional administration of niacinamide somatic consequences of I3–AA failed to develop and only changes in behavior were noted. If the picture obtained with the rats was due to the antimetabolite action of I3–AA, then it resembled the syndrome described by Jolliffe et al., who reported that the effects of acute niacinamide deficiency might be manifested solely by cerebral changes.

SUMMARY

The daily feeding of 1 to 5 mg amounts of indole 3-acetic acid (I3–AA) was found to produce definite changes in the behavior of rats within a minimum period of 5 weeks. The changes were characterized by lassitude, intractability on handling and a disregard for foreign objects introduced into their cages. These changes were found to be apparently irreversible in a majority of the affected rats after 10 weeks. At no time was there any weight loss.

The injection of I3–AA subcutaneously in guinea pigs produced a significant loss of righting reflex.

A similar affect was produced in guinea pigs by the injection of 6-aminonicotinamide when the animals were protected by an adequate amount of niacinamide.

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REPRODUCTION AND LACTATION STUDIES WITH BITCHES FED SEMIPURIFIED DIETS [1]

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INTRODUCTION

The nutritional requirements for reproduction in the bitch have not been clearly established. Campbell and Phillips ('52) found that a corn-soybean meal-alfalfa diet was inadequate for reproduction without supplemental vitamin B_{12}. The addition of vitamin B_{12} increased the number of pups born and decreased mortality. Campbell ('51) reported reproductive failure in the bitch fed a semipurified diet and the reproductive performance was favorably improved by the daily supplemental feeding of small amounts of fresh liver. In both studies there were reports of death losses occurring within the first 48 hours after birth. These data suggested that other factors were needed for the overall success in the reproductive cycle of the bitch during pregnancy, parturition and lactation. The caloric requirements of the growing dog have been studied, but these studies did not include reproduction (Cowgill, '28; Brody, Procter and Ashworth, '34; Morgan and Garrison, '33; Arnold and Elvehjem, '39; James and McCay, '50). The energy intake has been studied in the lactating rat but not in the dog (Slonaker, '27; Murray, '41). Lactating rats increased their dietary intake two- to three-fold over adult maintenance.

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This report presents data obtained with bitches fed semipurified diets through pregnancy, parturition and lactation and the effects of protein supplements and food intakes during the reproductive cycle.

EXPERIMENTAL

The studies in this series of experiments were made with mature beagle and cocker spaniel bitches. They were housed individually in cages with expanded metal floors and with access to food and water at all times. Experimental allotments in all cases were based upon age, thrift, size, breed and previous dietary history. Precautionary steps were taken to provide protection against canine distemper, infectious hepatitis and internal parasites.

The basal diet used in these studies was a semipurified ration composed of alcohol-extracted casein 20; sucrose 66; cotton seed oil 8; salts IV (Hegsted et al., '41, modified to include 2.5 mg of $NaMoO_4$ and 4.5 mg of $Co(NO_3)_2 \cdot 6H_2O$/100 gm of ration) 4 and 2 parts of a vitamin mix dried on sucrose. The vitamin mixture provided: thiamine 3.3, pyridoxine 3.3, riboflavin 3.3, niacin 10.0, menadione 9.2, calcium pantothenate 20.0, folic acid 0.3, biotin 0.2, vitamin B_{12} 0.02 and inositol 100.0 mg/kg of diet. The fat-soluble vitamins were given weekly in the form of halibut liver oil at the rate of 10 drops/dog. The oil was fortified by the addition of α-tocopherol at the rate of 50 mg/ml. The ration contained 0.1% of choline chloride.

The initial study was to determine whether protein supplements would improve reproductive performance of the bitch. The control lot was fed the basal ration only. Lot 2 animals were fed the basal diet plus 10% of fresh liver daily. Lot 3 bitches were fed 5% of additional protein (casein) displacing an equivalent quantity of sucrose. The lot 4 animals were fed 2% of liver fraction L,[2] displacing 2% sucrose. The animals in lot 5 were fed a ration the same as those in lot 3 except that the supplemental protein was autoclaved egg white.

[2] ''The 70% alcohol-insoluble fraction of the total aqueous extraction of raw liver subjected to enzymic action.'' Wilson and Co., Chicago, Illinois.

Consumption records as well as body weight records were kept. Matings were made according to good kennel practice.

An attempt was made to evaluate the role of methionine as a key dietary substance when it became evident that protein supplements in the initial study resulted in a marked improvement of reproduction. Three lots of 8 to 10 bitches each, some recruited from the first study, were allotted to the following dietary groups: Lot 1, basal ration only; lot 2, basal plus 0.30% of DL-methionine, lot 3, basal ration with 5% alcohol-extracted casein substituted for 5% sucrose. The husbandry practices used were described above.

RESULTS

The initial study extended over a two and one-half year period. During this period no evidence of unthriftiness appeared. Hair coats were normal and consistency of stools was good. The basal diet was a low-fiber diet with a moderately high energy content and was readily digestible.

Data pertinent to the study are summarized in table 1. It is clearly evident that the basal ration was inadequate for reproduction as indicated by the incidence of mortality. Further inspection of these data indicated that reproductive failure expressed itself in lower birth weight or inadequate nutrition, or a combination of both, during the nursing phase of reproduction. Since death was most frequent within the first 48 hours after birth, it would seem to reflect lowered vitality in the newborn. If this were true, the effects were produced by prenatal causes. Supplements of liver, casein, liver L fraction, or heated egg white protein reduced mortality losses, stimulated intrauterine growth as shown by birth weight, and increased the number of litters and pups weaned. The results obtained in the second study supported those of the first in that the basal ration was inadequate for reproduction, and that the addition of 5% of casein to the diet decreased the mortality rate while the animals fed the DL-methionine supplement showed no improvement in this respect over those fed the basal ration only.

TABLE 1

The effect of protein supplements on reproduction and lactation performance in the bitch

Experiment 1

LOT	SUPPLEMENT	LITTERS BORN	LITTERS WEANED	AV. WT./PUP		PUPS BORN	PUPS WEANED	MORTALITY
				Birth	35 Days			
				gm	*gm*			%
1		5	2	197	990	26	10	62
2	10% fresh liver	5	4	263	1003	24	15	35
3	5% casein	5	5	238	987	27	21	22
4	2% liver L [1]	3	3	246	863	15	11	27
5	5% egg white	5	5	220	956	32	22	31

[1] See text footnote 2, page 212.

Data on the food consumption of pregnant and lactating bitches are presented in table 2. These data showed that the dietary demands of the pregnant bitch were only slightly affected during the first half of pregnancy. Beginning with the 4th week the amount of food eaten began to increase and reached the highest intake during the 6th week. During the last week of pregnancy, food intake declined to maintenance levels. With the onset of lactation, dietary intake rose steadily until the maximum was reached by the 5th week. The intake level returned to maintenance ranges during the 8th

TABLE 2

Average food ingestion of 8 bitches during gestation and lactation [1]

GESTATION WEEK	FOOD/WK.	LACTATION WEEK	FOOD/WK.
	kg		*kg*
1	1.01	1	1.71
2	1.02	2	2.46
3	0.96	3	2.88
4	1.19	4	3.18
5	1.63	5	3.32
6	1.63		
7	1.21		
8	0.93		

[1] The average food intake of these dogs for maintenance was 0.98 kg/wk. Their average weight was 9.1 kg.

week. It was evident that appetite demands of the lactating bitch apparently reached the physiological limits of food intake as it approached 3.5 times the maintenance level. These results were confined to data from those bitches nursing 5 or more pups. It was found necessary with nursling pups to supplement their diet in order to maintain uniform and steady growth rates during the period between the 5th week and weaning at 8 weeks.

The effect of litter size upon the food intake of the lactating bitch is summarized in table 3. The weaning weight of young pups confined largely to their mothers' milk was reduced as the litter size increased above three. Litters of 4 pups or less

were successfully raised by mothers whose food intake was increased two-fold. It required a food intake increase of 2.5 to 3.0 times the maintenance level to enable mothers to suckle successfully litters of 4 to 8 pups to weaning. The dietary intake was critical in the 3rd week with litters of 6 or more pups, and during the 4th and 5th weeks with litters of 5 or less.

TABLE 3

Food consumption of the bitch as influenced by litter size

NO. OF BITCHES AVERAGED	PUPS WEANED	AV. WEANING WT./PUP	AV. FOOD INTAKE/ BITCH/WEEK	
			Maintenance	Lactation
		gm	*kg*	*kg*
1	8	911	1.06	3.03
3	6	967	1.03	2.88
5	5	983	0.94	2.54
5	4	948	0.95	1.97
3	3	1327	0.88	1.87
3	2	1208	0.88	1.69
3	1	1328	0.87	1.27

[1] Food intake average throughout the 5-wk. lactation period.

The basal diet used in these studies was estimated to contain 427 Cal./100 gm of ration. This diet was a low-fiber ration that was composed of ingredients with a high degree of digestibility. The records for the bitches fed this ration revealed little or no loss of body weight during lactation. In contrast, bitches of similar breeding and size fed our stock kennel ration which was estimated to contain 310 Cal./100 gm of ration lost weight during lactation when nursing litters of 4 or more pups.

DISCUSSION

The improved reproduction reported by Campbell ('51) as the result of supplementing a semipurified diet with fresh liver might be explained in two ways, among others. In as much as liver contributes a large variety of dietary factors, perhaps it contributed an unknown dietary factor needed for reproduction, or some known nutrient present in marginal

amounts was added by liver in an amount sufficient to meet the needs for reproduction. The data from the present studies support the latter interpretation. In these studies, it was demonstrated that supplemental casein or heated egg white improved the reproductive performance of the bitch in that more vigorous pups were born and survival was greatly increased. This view of the problem and its interpretation are in line with the classic studies culminating in a recent report by Schultze ('57) where he was able to show that the addition of nonessential amino acids to a diet containing ample amounts of the essential amino acids greatly improved reproductive performance of the female rat.

Since mortality data indicated that death losses occurred largely within 48 hours of birth and the mean birth weights were increased when supplemental protein was fed, it seems likely that high mortality occurred as a result of malnutrition in utero rather than because of a lactation defect. It has been shown that semipurified diets containing 20% of casein were improved by cystine and by methionine supplements in the growing rat (Pavcek and Baum, '41; Mulford and Griffin, '42). The addition of methionine to the basal ration in these studies did not improve reproduction of the bitch.

SUMMARY

A study of certain dietary effects upon pregnancy, parturition and lactation in the bitch has been made. Bitches fed the semipurified basal ration bore pups with lower body weights at birth and a decreased survival rate up to 48 hours. The site of interference appeared to be intrauterine with a subclinical fetal malnutrition. Supplementary protein, fresh liver or liver L improved birth weight and greatly increased survival to weaning. The addition of methionine was without beneficial effect. It has been shown that the food intake demand of the pregnant bitch increased at mid-pregnancy, that it increased progressively with each week of lactation to a peak during the 4th or 5th week. The upper physiological limit of food intake was nearly 3.5 times the maintenance level;

this increase in demand for food was associated with the number of nurslings suckled. Litters larger than 4 subjected the lactating bitches used in these studies to a marked stress, due to their difficulty in eating sufficient food.

ACKNOWLEDGMENT

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EVIDENCE FOR AN UNIDENTIFIED FACTOR NECESSARY FOR MAXIMUM EGG WEIGHT IN CHICKENS [1]

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The need of the mature fowl for unidentified dietary factors has been the subject of investigation in this laboratory for some time. Recently reported were results showing that egg production, egg weight and hatchability of fertile eggs were unaffected when either a corn-soy diet or a fish meal-sucrose semi-purified diet was supplemented with various sources of unidentified chick growth factor (Jensen and McGinnis, '57). A significant increase in egg production and egg weight was obtained, however, with the corn-soy basal as compared with the semi-purified basal diets. In a previous experiment Patterson ('55) obtained a significant improvement in both egg weight and egg production with birds fed a practical diet as compared with those fed a purified diet consisting principally of isolated soybean protein [2] and sucrose. No significant difference in hatchability of fertile eggs was obtained. Waibel et al. ('55) also demonstrated a significantly greater egg weight and egg production with birds fed a corn-soy diet compared with those fed an alpha-protein-glucose diet.

These findings indicate that an unidentified nutrient was missing from the purified diets. A study was undertaken to investigate further the differences between purified and prac-

[1] Scientific paper no. 1672, Washington Agricultural Experiment Stations, Pullman. Project no. 1247. This investigation was supported in part by funds provided for biological and medical research by the State of Washington Initiative no. 171.

[2] The Drackett Products Company, Cincinnati, Ohio.

tical diets for the mature fowl. One possibility considered was a deficiency in protein or an amino acid, because it is well known that these deficiencies readily affect egg size (Byerly et al., '33). Another consideration was that the corn or soybean meal contained in the practical diets possessed an unidentified factor necessary for maximum egg weight and egg production in chickens. The following experiments were conducted to test these hypotheses. Evidence will be presented to show that crude corn oil contains an unidentified nutritional factor for the hen.

PROCEDURE

Experiment 1 — Effect of protein levels and different carbohydrates

Single-Comb White Leghorn pullets, approximately 8 months old, were randomly distributed into 12 pens of 20 birds each. They were housed in 4′ × 8′ cages with wire floors. Three White Leghorn cockerels were placed in each pen. Records were kept on egg production, egg weight and hatchability. All eggs laid were weighed, except those having double yolks or no yolk, and those with broken shells.

The treatments consisted of two levels of protein (15 and 20%). Each was fed with three different sources of carbohydrate (glucose, cornstarch and yellow corn). The composition of the 15% protein diet containing glucose is presented in table 1. Cornstarch was substituted completely for the glucose. With the substitution of yellow corn, the amount of fish meal was lowered in order to maintain a constant level of protein. The 20% protein diet was made by increasing the level of fish meal. In all cases where the level of fish meal was altered, appropriate changes were also made in the amounts of calcium and phosphorus to keep these constant. The quantities of all other ingredients remained constant for all treatments.

All birds were placed on a stock diet consisting largely of yellow corn, soybean oil meal and fish meal for two weeks before placed on the experimental diets. Two pens were used for each treatment. The birds were kept on the experimental

diets for 8 weeks and then transferred to the different treatments as shown in table 2. Each replicate was placed on a different carbohydrate from that which had previously been fed, but received the same protein level. The birds were maintained on the new treatments for three weeks, after which they were returned to the stock diet for 4 weeks.

TABLE 1

Composition of basic 15% protein diet

INGREDIENT	AMOUNT
	%
Fish meal, herring (73% protein)	20.800
Glucose [1]	68.870
Cellulose [2]	3.000
NaCl (iodized)	0.400
KCl	0.400
$MgSO_4 \cdot 7H_2O$	0.250
$MnSO_4$ (25.5% Mn)	0.050
Steamed bonemeal	1.500
Limestone	3.100
Choline chloride (70%)	0.125
Vitamin mixture [3]	1.000
Mineral mixture [4]	0.500

[1] Cerelose.

[2] Solka Floc, The Brown Company, Berlin, N. H.

[3] Supplied per pound of feed: niacin, 15 mg; Ca pantothenate, 8 mg; pyridoxine·HCl, 2.6 mg; riboflavin, 2.0 mg; thiamine·HCl, 5.0 mg; folie acid, 0.3 mg; biotin, 0.08 mg; vitamin B_{12}, 0.004 mg; vitamin A, 3000 I.U.; vitamin D, 750 I.C.U.; *d*-alpha-tocopheryl acetate, 15 mg; menadione, 0.5 mg.

[4] Supplied per pound of feed: $FeSO_4 \cdot H_2O$, 100.0 mg; $ZnSO_4$, 4 mg; $C_0Cl_2 \cdot 6H_2O$, 1 mg; $Cu\ SO_4 \cdot 5H_2O$, 7 mg.

During the latter part of the first 8-week experimental period, a week's collection of eggs from each pen was used for measurement of yolk size. Following autoclaving for 10 minutes at 15 pounds pressure, all eggs were weighed and the yolks were removed and weighed. Also during the latter part of the first 8-week experimental period, a sample of 12 eggs from each pen was used to determine yolk cholesterol content by the method of Cook and Mehlenbacher ('46).

TABLE 2

Effect of dietary protein level and carbohydrate source on egg weight

PEN NO.	AV. PRE-EXPERIMENTAL EGG WEIGHT [1]	FIRST TREATMENT				SECOND TREATMENT				AV. POST-EXPERIMENTAL EGG WEIGHT
		Protein	Carbohydrate	Egg weight [2]		Protein	Carbohydrate	Egg weight [3]		
				Av.	% base			Av.	% of first treatment av.	
	gm	%		*gm*		%		*gm*		*gm*
6	54.0	15	Glucose	54.8	101.5	15	Starch	54.0	98.5	58.8
7	53.9	15		55.1	102.2	15	Corn	58.0	105.3	59.0
Av.	54.0			55.0	101.9					
9	51.8	15	Starch	52.0	100.4	15	Glucose	52.6	101.2	55.7
11	52.7	15		52.1	98.9	15	Corn	55.6	106.7	56.5
Av.	52.2			52.1	99.8					
2	52.7	15	Corn	55.3	104.9	15	Starch	54.6	98.7	56.2
3	52.3	15		55.0	105.6	15	Glucose	53.6	97.5	55.8
Av.	52.5			55.2	105.1					
10	53.4	20	Glucose	52.7	98.7	20	Starch	52.1	98.9	56.0
12	52.1	20		52.0	99.8	20	Corn	54.2	104.2	55.4
Av.	52.7			52.4	99.4					
4	53.2	20	Starch	53.5	100.6	20	Glucose	53.6	100.2	56.3
8	51.8	20		53.9	104.1	20	Corn	55.9	103.7	57.8
Av.	52.5			53.7	102.3					
1	52.3	20	Corn	54.9	105.0	20	Starch	54.3	98.9	56.5
5	53.3	20		55.2	103.6	20	Glucose	55.3	100.2	58.1
Av.	52.8			55.1	104.4					

[1] Stock diet for two weeks.

[2] Last 6 weeks of 8-week experimental period.

[3] Entire three-week experimental period.

The effect of the various dietary treatments on egg weight is given in table 2. During the first 8-week experimental period, a marked increase in egg weight was observed only with the diets containing corn. This was true at both protein levels. Increasing the protein level from 15 to 20% did not bring about maximum egg weight in the absence of corn. Following the transfer to the new experimental treatments for three weeks, all the birds now receiving corn produced eggs with markedly increased weight, whereas there was no change, or a decrease, in egg weight in the pens now fed either cornstarch or glucose as the carbohydrate. When the birds were placed on the stock diet at the end of the experimental period, there was a rapid increase in egg size in all cases in which corn had not been fed previously.

During the first 8-week experimental period at both levels of protein, egg production in the birds not receiving corn tended to decrease as the experiment progressed (figs. 1 and 2). When changed from either glucose or starch to corn, the rate of egg production increased, whereas in all other groups receiving glucose or cornstarch there was a decrease. After placing the birds on the stock diet, all groups not previously receiving corn showed a marked increase in egg production, whereas little change occurred with those previously receiving corn.

There was little difference in the hatchability of fertile eggs among the various treatments for eggs laid during the third week on the experimental diets (table 3). Results obtained with eggs laid during the 7th week, however, showed a lower level of hatchability with all treatments not including corn. After the transfer to new treatments, all the birds not receiving corn in the new treatments or in the previous treatments produced fertile eggs with a greatly reduced hatchability. The hatchability of eggs laid by birds that received corn in the previous experimental diets, but cornstarch and glucose in the new experimental treatments, was higher than for those receiving glucose and cornstarch throughout the two experimental periods. This indicated that a factor was stored

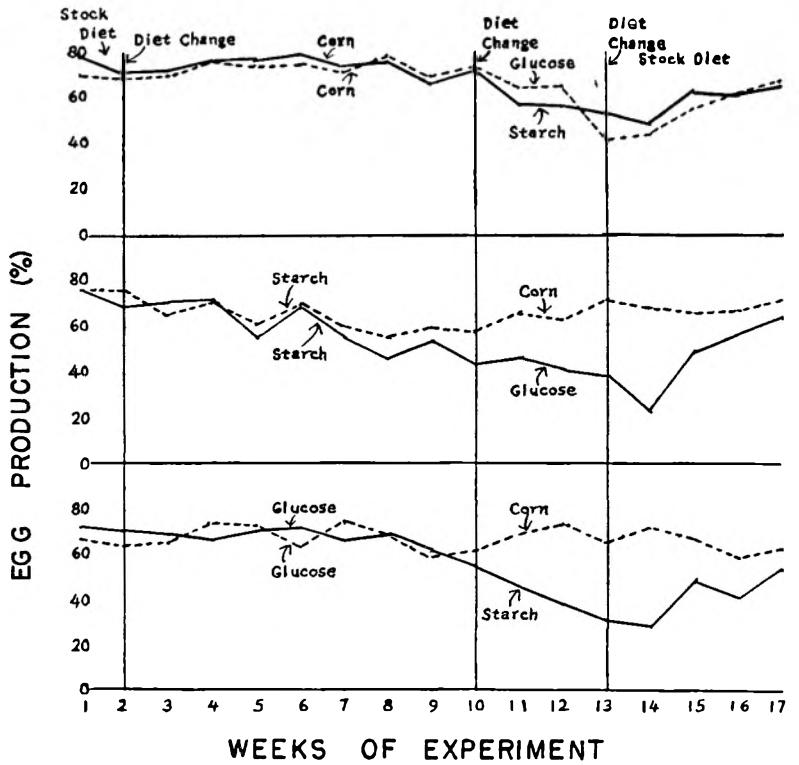

Fig. 1 Effect of carbohydrate on rate of egg production by chickens fed diets containing 15% of protein.

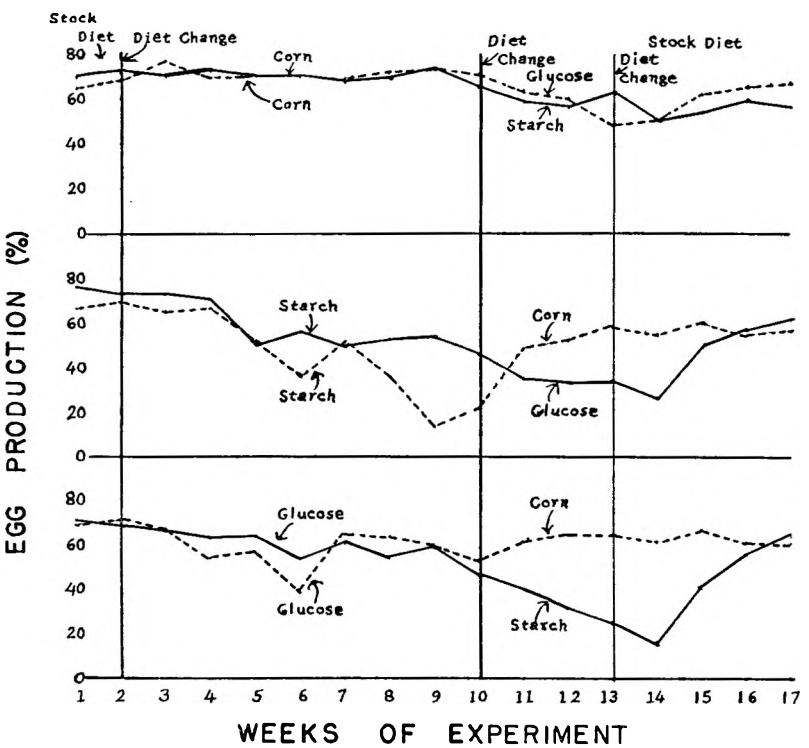

Fig. 2 Effect of carbohydrate on rate of egg production by chickens fed diets containing 20% of protein.

in the body of the hens which was not depleted during the second experimental period.

The ratio of yolk weight to whole egg weight was relatively constant within the various treatments (table 4). This suggested that the primary effect of the unidentified factor in corn was on yolk size rather than on albumen or shell weight. It is well known that the size of the yolk largely determines the final size of the egg. The cholesterol content of the yolk was not greatly affected by the dietary treatments (table 4).

Experiment 2 — Effect of dehydrated grass and levels of corn

Because it had been shown in the previous experiment that corn was a source of an unidentified factor necessary for maxi-

TABLE 3

Effect of dietary protein level and carbohydrate source on hatchability of fertile eggs

FIRST TREATMENT					SECOND TREATMENT		
PEN NO.	Protein	CHO	Hatchability [1] 5th week	Hatchability [1] 9th week	Protein	CHO	Hatchability 13th week
	%		%	%	%		%
6	15	Glucose	97.4	86.2	15	Starch	76.9
7	15		89.1	71.2	15	Corn	98.7
Av.			93.3	78.7			
9	15	Starch	91.9	85.7	15	Glucose	69.2
11	15		96.3	82.8	15	Corn	97.4
Av.			94.3	84.3			
2	15	Corn	96.3	94.7	15	Starch	93.2
3	15		88.2	88.7	15	Glucose	92.3
Av.			92.2	91.7			
10	20	Glucose	92.6	90.5	20	Starch	55.0
12	20		94.2	81.8	20	Corn	95.5
Av.			93.0	86.2			
4	20	Starch	90.9	90.6	20	Glucose	58.6
8	20		94.8	78.6	20	Corn	100.0
Av.			92.9	84.6			
1	20	Corn	98.4	96.3	20	Starch	86.0
5	20		92.5	100.0	20	Glucose	94.4
Av.			95.5	98.2			

[1] Data on hatchability are for the third and 7th weeks of the experimental period.

mum egg weight and egg production in chickens, another experiment was undertaken to determine if a lower level of corn would bring about the same effect. In addition, dehydrated grass was tested as a source of the factor.

Single-Comb White Leghorn pullets approximately 10 months old were divided randomly into 4 pens of 15 birds each. They were housed in individual cages with wire floors. Egg

TABLE 4

Effect of dietary protein level and source of carbohydrate upon yolk size and cholesterol content of yolks

PEN NO.	TREATMENT		AV. WEIGHT		YOLK FRACTION OF EGG	CHOLESTEROL CONTENT
	Protein	CHO	Egg	Yolk		
	%		*gm*	*gm*	%	%
6	15	Glucose	56.1	17.1	30.5	1.62
7			56.0	16.6	29.7	1.62
Av.			56.1	16.9	30.1	1.62
9	15	Starch	53.7	16.8	31.4	1.36
11			52.5	16.8	32.0	1.60
Av.			53.1	16.8	31.7	1.48
2	15	Yellow	57.3	17.0	29.8	1.58
3		corn	57.2	17.3	30.4	1.48
Av.			57.3	17.2	30.1	1.53
10	20	Glucose	53.9	16.1	29.8	1.40
12			51.8	15.6	30.2	1.64
Av.			52.9	15.9	30.0	1.52
4	20	Starch	55.1	16.4	29.6	1.61
8			54.3	15.4	28.8	1.30
Av.			54.7	15.9	29.2	1.46
1	20	Yellow	55.7	16.2	29.2	1.48
5		corn	55.1	16.6	30.1	1.63
Av.			55.4	16.4	29.7	1.56

production records were kept and all eggs laid were weighed (with the same exceptions as in experiment 1). During the first three weeks all the birds were fed a stock diet, following which they were placed upon the experimental treatments listed in table 5. The basal diet was the same as shown in table 1, except that protein level was adjusted to approximately 20%. After a period of three weeks the hens were again placed on the stock diet.

A marked increase in egg weight was obtained only with the diet containing 70% of corn (table 5). Following the return to the stock diet, the eggs laid by all birds not previously receiving 70% of corn greatly increased in size. Egg production was maintained at the highest rate by the birds receiving the 70% corn diet, but all groups dropped to relatively low rates during the post-experimental period.

TABLE 5

Egg weight and rate of egg production as affected by dehydrated grass and corn supplementation of a fish meal-glucose basal diet

SUPPLEMENT	AV. EGG WEIGHT (WKS.)			AV. EGG PRODUCTION (WKS.)		
	1–3 [1]	4–6 [2]	9–11 [1]	1–3 [1]	4–6 [2]	9–11 [1]
	gm	*gm*	*gm*	*%*	*%*	*%*
None	53.5	53.2	57.8	63.2	56.7	45.4
5% dehydrated grass	52.8	53.3	57.7	53.3	51.1	38.1
70% yellow corn	54.8	56.9	57.2	65.7	63.8	42.9
5% yellow corn	54.3	53.2	58.7	66.0	45.7	35.2

[1] Fed stock diet during this period.

[2] Fed experimental diets during this period.

Experiment 3 — Distribution of egg size factor in various feed ingredients

The results of experiment 2 showed that a relatively large amount of corn was necessary in the diet to improve egg size and that 5% of dehydrated grass was a relatively poor source of the factor. A third experiment was conducted, therefore, to study the effect of different levels of yellow corn and also, the addition of other ingredients such as barley, soybean oil meal and animal tallow. Furthermore, the effectiveness of several fractions of corn was investigated.

Half of the birds used in this experiment were the same as those used in experiment 1. The other half of the birds had been on an experiment unrelated to the present study. They were of the same strain and age, however. All the birds were fed the stock diet for 4 weeks. Then the new experimental

treatments were randomly assigned to two pens per treatment (table 6). The basal diet was the same as that used in experiment 2. In the treatment involving soybean oil meal this protein completely replaced fish meal in the basal diet. The various fractions of corn were fed at levels approximating the content of these fractions in whole corn when included in the ration at a level of 70%.

The highest level of corn was necessary to maintain egg weight at its maximum level (table 6). Barley at the 70% level was only as effective as 20 to 45% of corn in the diet; soybean oil meal was almost as effective as 70% of corn. At the levels fed, corn bran, dried corn steep liquor, and corn oil meal

TABLE 6

Effect on egg weight in chickens of the addition of corn, barley, soybean meal, tallow and corn fractions to a fish meal-glucose basal diet

SUPPLEMENT	FIRST EXPERIMENT AVERAGE EGG WEIGHT			CONFIRMATORY EXPERIMENT AVERAGE EGG WEIGHT		
	Pre-experimental [1]	Experimental [2]	% of base	Pre-experimental [1]	Experimental [3]	% of base
%	*gm*	*gm*		*gm*	*gm*	
None	56.8	54.7	96.3	55.3	55.9	101.0
Yellow corn, 20	56.2	55.0	97.9	—	—	—
Yellow corn, 45	55.7	55.0	98.7 [4]	—	—	—
Yellow corn, 70	57.6	57.9	100.5 [5]	55.1	57.8	104.9 [4]
Barley, 70	57.3	56.4	98.5 [4]	55.7	57.7	103.6 [4]
Soybean oil meal, 40	56.6	56.3	99.5 [5]	56.6	59.4	104.9 [4]
Corn bran, 5	56.5	54.6	96.6	—	—	—
Corn steep liquor (dry), 5	57.2	55.0	96.2	—	—	—
Corn oil meal, 15	56.4	54.9	97.4	—	—	—
Corn gluten meal, 15	57.6	57.0	98.9 [4]	—	—	—
Crude corn oil, 3	57.6	57.6	100.0 [5]	54.9	57.6	104.9 [4]
Animal tallow, 3	58.3	55.1	94.6	55.5	56.8	102.2

[1] Last two weeks of a period when fed stock ration.

[2] Last two weeks of 5-week experimental period.

[3] Last week of 4-week experimental period.

[4] Significantly different from basal diet ($P = < 0.05$), using Duncan's ('55) multiple range tent.

[5] Significantly different from basal diet ($P = < 0.01$), using Duncan's ('55) multiple range test.

were largely ineffective in maintaining egg weight. Corn gluten meal (15%) was equivalent to about 45% of corn. Crude corn oil (3%) was as effective as 70% of corn, but another fat source, animal tallow (3%) was completely ineffective.

Experiment 4 — Corroborative experiment on the potency of certain feed ingredients as a source of the unidentified factor for egg size

The results obtained in experiment 3 indicated that crude corn oil was a potent source of the unidentified factor necessary for maximum egg weight. The present experiment was undertaken to determine whether this observation could be repeated and to observe again the effectiveness of barley, soybean oil meal and animal tallow. The same birds as those used in experiment 3 were employed in this experiment. All birds were placed upon the basal diet for 4 weeks in order to deplete them of the unidentified factor. At the end of this period the experimental treatments were randomly assigned to three pens of 20 birds each and the birds were kept on the experimental diet for 4 weeks. As in previous experiments, all eggs laid were weighed except those having double yolks or no yolks, and those with broken shells.

Corn, crude corn oil and soybean oil meal markedly improved egg weight (table 6), whereas 3% of animal tallow again failed to improve egg weight markedly. Barley contained the unidentified factor, but was not as effective as the corn or corn crude oil in raising egg size. These results, therefore, confirm those obtained previously.

DISCUSSION

The results reported show that an unidentified factor, present in crude corn oil, yellow corn, and certain other materials, was necessary for maximum egg weight in the chicken. The results also indicated that the factor necessary for maximum rate of egg production and hatchability appears to be of plant

origin because of the response obtained when sources of the factor are added to a diet containing relatively high levels of fish meal. Furthermore, animal tallow failed to give a response. The response does not seem to be related to protein or amino acids; raising the level of protein from 15 to 20% did not affect the results, and 3% of crude corn oil gave a maximum response. Increases in energy level cannot account for the response because complete substitution of soybean oil meal for fish meal (which greatly reduced the concentration of available energy) significantly improved egg weight in two experiments. Furthermore, Jensen and McGinnis ('52) reported that eggs were not significantly different in weight from hens fed diets with a wider variation in productive energy (Fraps, '46) level than was the case when 3% oil was added to the basal diet in the present experiments. It is possible that the factor may be fat-soluble since it is concentrated in the corn oil.

A large number of materials have been shown to improve the growth rate of chicks and poults under a variety of conditions. Unidentified growth factors have been reported in such materials as fish meal, fish solubles, distillers' dried solubles, dried brewers yeast, grass juice, alfalfa meal, dried whey, peanut meal and others. Whether the various growth responses are due to a single factor or to a multiplicity of factors has been discussed by many investigators and evidence has been presented for the existence of three different unidentified growth factors (Kohler and Graham, '51; Fisher et al., '54; Combs et al., '54; Norris, '54; and Scott et al., '54).

In relating these factors to the unidentified factor shown in this study to be necessary for maximum egg weight, it appears that this factor is not present in large quantities in most of the materials mentioned. In a previous report from this laboratory (Jensen and McGinnis, '57) no improvement in egg weight was obtained by supplementing the diet with either distillers' dried solubles or a grass juice concentrate. The fact that the basal diet contained a relatively high level of fish meal would probably eliminate the "fish" factor. In

the present study, dehydrated grass failed to affect egg weight.

Denton et al. ('54) observed that the addition of egg yolk to a corn-soy type of ration markedly improved the growth rate in chicks. This has been confirmed by Hopper et al. ('56) and Arscott ('56). It would seem that egg yolk might be a source of the factor required for maximum egg weight. The evidence obtained in the present report, however, that both corn and soybean oil meal are sources of the egg weight factor, suggest that the growth factor(s) in egg yolk may not be identical with the egg weight factor. Carver and Johnson ('53, '54) reported that wheat germ oil, corn oil, soybean oil and an oleic acid concentrate were sources of growth factors for chicks. It is possible that the factor(s) producing this growth effect may be identical with the one affecting egg weight. Further studies will be required on distribution and fractionation of the egg weight factor in order to determine its relationship with other reported unidentified factors.

In view of the report by Beveridge et al. ('55), that in humans corn oil led to lower plasma cholesterol levels than rations devoid of fat, or containing animal fat, it was of interest to determine the effect of different diets used in the present study on cholesterol deposition in the egg. No important difference, however, was observed in cholesterol levels in the yolks laid by hens receiving the different diets. This was true even for the diet containing 70% of corn which would have contained somewhat over 2.5% of corn oil. In other determinations not reported, it was found that cholesterol deposition in the yolk was not affected by the addition of 3% of crude corn oil to the basal diet.

The results in the present study differ somewhat with respect to hatchability of fertile eggs from the previous report from this laboratory (Jensen and McGinnis. '57). A marked difference in hatchability was obtained among the dietary treatments, but in the previous study no statistically significant difference was observed between a practical and semi-purified diet. The reason for this is not apparent.

SUMMARY

Evidence is presented that an unidentified factor present in yellow corn and in certain other feedstuffs is necessary for maximum egg weight, egg production, and hatchability of fertile eggs in the chicken. Feeding of graded levels of yellow corn showed that a dietary level of 70% was necessary to obtain the maximum effect. Dehydrated grass was not a good source of the factor. Several fractions of corn were tested and the factor was found to be concentrated in the crude corn oil fraction. A level of 3% of crude corn oil was as effective as 70% of corn in raising egg weight, whereas 3% of animal tallow was ineffective. A deficiency of the unidentified factor apparently primarily affected the quantitative deposition of yolk rather than the deposition of albumen or shell. Deposition of cholesterol in the yolks of the eggs was not affected by the presence of concentrated sources of the unidentified factor.

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THE INFLUENCE OF VARIED CELLULOSE AND NITROGEN LEVELS UPON RATION DIGESTIBILITY AND NITROGEN BALANCE OF LAMBS FED SEMIPURIFIED RATIONS[1]

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The provision of an efficient, high roughage ration for ruminants requires a consideration of the ruminant's alimentary flora and fauna requirements in addition to the animal's tissue requirements. Through the action of these alimentary microorganisms, a significant portion of the relatively undigestible plant polysaccharides is degraded and fermented, thereby yielding products useful to the animal (Baker and Harris, '47; Carroll and Hungate, '54). Various factors and conditions have been shown to influence the extent of this degradation, both *in vitro* and *in vivo*.

Mitchell et al. ('40), Hamilton ('42) and others (Lindsey and Smith, '10; Ewing and Wells, '15; Armsby and Fries, '18; Briggs, '37), have demonstrated the depressing effect of soluble carbohydrates upon crude fiber digestibility. Conversely, Williams ('25) and others have shown that low levels of soluble carbohydrates increase the digestibility of fiber. Cox ('48) has indicated that rate of gain and feed efficiency in fattening lambs may be related to a balance between total digestible nutrient intake and crude fiber intake. Rations having a crude fiber-total digestible nutrient ratio of 1 to 3.8 and containing approximately 18% of crude fiber

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gave maximum gain and feed efficiency. Axelsson ('39a,b, '40a) presented evidence that the metabolizable energy of dairy rations was most efficiently used if they contained 18 to 23% of crude fiber and 10 to 18% of crude protein. Schulze ('55) also has concluded that dairy rations containing 18% of crude fiber are optimum for milk production in cattle. The addition of soybean meal increases the digestibility of the dry matter of poor-quality, low-protein corn cob rations, and partially reverses the depressing effect of starch on the digestibility of dry matter, according to Burroughs and Gerlaugh ('49) and Burroughs et al. ('50). These results suggest that an optimum nutritive ratio, an optimum ratio of soluble to insoluble carbohydrate, and a minimum nitrogen level may exist for the digestibility of individual nutrients and fibrous components of ruminant rations.

The purpose of this experiment was to study the interrelated effects of varied cellulose and nitrogen levels upon ration digestibility and nitrogen balance by lambs. Semipurified rations were used in order that cellulose and nitrogen levels could be altered with a minimum change in other ration components. Also the "envelopment" effect (see Lancaster, '43) of cellulose was eliminated in that relatively pure sources of cellulose and nitrogen were used.

METHODS

Three semipurified basal rations were compounded to contain 25, 37 and 49% of cellulose by replacing starch with wood pulp.[2] These basal rations were treated with water, autoclaved, and dried as previously described (Ellis et al., '56) to reduce dustiness and improve their palatability. Prior to twice-daily feeding, each basal ration was combined with one of three nitrogen-vitamin supplements containing either 10, 12.5 or 15% of nitrogen. Nitrogen supplements were formulated by diluting purified soybean protein[3] with starch

[2] Solka Floe (BW-100), The Brown Company, Berlin, New Hampshire.

[3] Trade name: Drackett, C-1 assay protein. Obtained from the Drackett Company, Cincinnati 32, Ohio.

to yield the desired percentage of nitrogen. A daily ration consisted of 672 gm basal ration and 128 gm nitrogen-vitamin supplement. The three basal rations combined with the proper nitrogen-vitamin supplement resulted in a daily ration containing either 21.4, 31.4 or 41.6% of cellulose and 1.65, 2.05 or 2.45% of nitrogen by analysis (basal rations contained 0.6% of nitrogen). The compositions of the semipurified rations are shown in table 1.

Wether lambs of Colorado origin, weighing 65 to 80 pounds initially, were used in this experiment. All lambs were allowed three weeks to adjust to the semipurified rations prior to initiating the experiment. The experimental design allocated 12 lambs into two lots of 6 lambs each. Each lot of lambs was further divided into three groups of two lambs each. Each group within lot I was assigned a constant nitrogen level and

TABLE 1

Composition of experimental rations

INGREDIENT	RATION		
	C_1	C	C_3
	%	%	%
Wood pulp [1]	15.0	26.0	38.0
Starch	28.0	17.0	5.0
Beet pulp	33.8	33.8	33.8
Minerals [2]	5.0	5.0	5.0
Lard	2.0	2.0	2.0
Protein-vitamin supplement			
Soybean protein [3]-starch	16.0 [5]	16.0 [5]	16.0 [5]
Vitamin A and D [4]	0.2	0.2	0.2
	100.0	100.0	100.0

[1] Solka Floc (B.W. 100), The Brown Co., Berlin, New Hampshire.

[2] Mineral mixture of Ellis et al. ('56).

[3] Trade name: Drackett. Obtained from the Drackett Products Co., Cincinnati 32, Ohio.

[4] Stabilized vitamin A and D guaranteed to contain 2250 I.U. vitamin A and 400 I.U. vitamin D per gram. Obtained from Thompson Hayward Company, Kansas City, Missouri.

[5] The following ratios of Drackett protein to starch were employed to formulate the protein supplements for the N_1, N_2 and N_3 rations, respectively: 1: 1, 3: 1 and 1: 0.

variable cellulose levels; each group within lot II was assigned a constant cellulose level and variable protein levels. These assignments, as shown in table 2, were made on the basis of body weight following the three-week adjustment period.

During the 14-day adjustment periods following ration assignments, lambs were fed in individual feeding stalls twice daily and were together in a sheltered concrete lot the

TABLE 2

Schematic diagram of experimental design

ANIMAL NUMBER	ORDER OF EXPERIMENTATION			CONSTANT COMPONENT
	1	2	3	
		Lot I		
40	C_1[1]	C_2	C_3	N_1[2]
41	C_3	C_1	C_2	
43	C_1	C_2	C_3	N_2
44	C_3	C_1	C_2	
47	C_1	C_2	C_3	N_3
48	C_3	C_1	C_2	
		Lot II		
49	N_1	N_2	N_3	C_1
50	N_3	N_1	N_2	
51	N_1	N_2	N_3	C_2
52	N_3	N_1	N_2	
53	N_1	N_2	N_3	C_3
54	N_3	N_1	N_2	

[1] C_1 = 21.4% cellulose.
C_2 = 31.4% cellulose.
C_3 = 41.6% cellulose.

[2] N_1 = 1.65% nitrogen.
N_2 = 2.05% nitrogen.
N_3 = 2.45% nitrogen.

other 21 hours per day. Lambs were then transferred to metabolism stalls for three days before initiating the 6-day fecal and urinary collections according to procedures previously described (Ellis et al., '56).

Feed and feces were analyzed for nitrogen, ether extract, water and ash by the methods of the Association of Official Agricultural Chemists ('45). Cellulose was determined as described by Crampton and Maynard ('38). Fiber was determined as the organic residue from a one-hour digestion

with sulfuric acid (1 N) as described by Walker and Hepburn ('57). Such "fiber" has been shown by Walker and Hepburn ('55) to be more closely correlated with digestible energy than either cellulose or crude fiber determined by the official method.

The differences in coefficients of digestibility of ration components as affected by level of nitrogen and cellulose were analyzed by analysis of variance; the linear and quadratic effects of cellulose and nitrogen level upon digestibility were determined with the aid of a table of coefficients and divisors for sets of orthogonal comparisons and least significant differences between means ($P = 0.05$) were calculated utilizing a table of Q; all as described by Snedecor ('56). Simple and partial correlations were computed as suggested by Mills ('38).

RESULTS

Mean digestibility coefficients for the constituents of each ration are summarized in table 3. Each coefficient is a mean of separate determinations on 4 different lambs. A summary of the statistical analysis pertaining to digestibility shown in table 4.

Effect of cellulose on digestibility. Increasing cellulose levels significantly depressed the digestibility of nitrogen-free-extract ($P < 0.005$), organic matter ($P < 0.005$), and total digestible nutrients of the ration ($P < 0.005$). These effects were statistically linear ($P < 0.005$) with increasing cellulose levels although the depressive effect on nitrogen-free-extract also tended to be quadratic. There was no significant difference in the digestibility of the nitrogen-free extract of rations containing 21.4% (low) and 31.4% (medium) cellulose when these rations contained 1.65% (low) and 2.05% (medium) nitrogen. A significant difference ($P < 0.05$) in the digestibility of the nitrogen-free extract did exist between low and medium cellulose rations when they contained 2.45% of (high nitrogen) nitrogen.

TABLE 3

Mean composition and digestibility of semi-purified rations

RATION DESIGNATION [1]	N_1C_1	N_1C_2	N_1C_3	N_2C_1	N_2C_2	N_2C_3	N_3C_1	N_3C_2	N_3C_3	L.S.D. P = 0.05
Composition [2]										
Nitrogen, %	1.65	1.65	1.65	2.05	2.05	2.05	2.45	2.45	2.45	
Cellulose, %	21.4	31.4	41.6	21.4	31.4	41.6	21.4	31.4	41.6	
Fiber, %	23.7	33.7	43.9	23.7	33.7	43.9	23.7	33.9	43.9	
N.F.E., %	53.4	44.1	33.9	50.4	41.0	30.4	47.3	38.2	27.2	
Digestibility coefficient [3]										
Nitrogen	58.6	58.5	59.0	74.7	76.0	74.4	76.2	77.3	75.2	3.4
Cellulose	44.2	51.9	53.0	50.7	56.7	59.7	47.4	49.6	51.3	7.5
Normal acid fiber	40.1	48.8	49.4	47.5	54.1	56.7	42.5	48.9	48.2	6.9
N.F.E.	90.1	89.0	84.8	91.5	90.5	85.4	90.5	88.7	83.2	1.3
Ether extract	68.4	68.2	77.4	70.6	70.0	80.5	65.7	70.6	78.3	4.2
Organic matter	74.4	71.5	66.6	78.4	75.3	70.8	76.5	72.0	66.0	3.3
T.D.N.[4]	67.2	64.8	60.4	69.9	67.9	64.2	68.5	64.9	60.0	5.4
Nutritive ratio, 1:	10.1	9.7	9.0	6.3	6.0	5.7	4.9	4.5	4.2	

[1] Designations defined in footnote to table 2.

[2] Composition on an air dry basis. Each value is the mean composition of 4 separate ration samples. All rations contained approximately 2% ether extract and 86.5% organic matter.

[3] Apparent coefficients. Each digestibility coefficient is a mean of determinations on 4 different lambs.

[4] Determined as the sum of % digestible cellulose, % digestible N.F.E., % digestible nitrogen multiplied by 6.25, and % digestible ether extract multiplied by 2.25.

TABLE 4

Summary of statistical analysis pertaining to digestibility

SOURCE OF VARIATION	DEGREES OF FREEDOM	F RATIOS					
		Digestibility of					% Total digestible nutrients
		Organic matter	Cellulose	N-free extract	Ether extract	Nitrogen	
Cellulose level (C)	2	15.52 [2]	2.25	53.48 [2]	15.11 [2]	0.44	11.22 [2]
Linear effect	1	30.67 [2]	4.40 [1]	96.00 [2]	25.38 [2]	0.88	22.01 [2]
Quadratic effect	1	0.38	0.10	10.97 [2]	4.83 [1]	0.00	0.44
Nitrogen level (N)	2	3.44	2.50	3.63 [1]	0.75	58.43 [2]	2.75
Linear effect	1	0.10	0.50	0.61	0.00	92.21 [2]	0.02
Quadratic effect	1	6.78 [1]	4.50 [1]	6.66 [1]	1.51	24.65 [2]	5.48 [1]
Constant N vs. constant cellulose (CN)	1	0.02	1.34	0.14	0.39	3.18	0.00
Interactions							
C × N	4	0.16	0.12	0.46	0.38	1.47	0.16
N × CN	2	0.84	0.72	1.20	1.76	3.34	0.76
C × CN	2	2.25	1.60	1.60	1.36	1.47	2.97
C × N × CN	4	0.44	0.47	0.65	0.47	1.30	0.55
Error	18						

[1] Statistically significant, P = 0.05.

[2] Statistically highly significant, P = 0.005

Cellulose and ether extract digestibility were significantly linearly increased by elevations in cellulose levels ($P < 0.05$ and $P < 0.005$, respectively). Varying cellulose levels affected the digestibility of "normal acid fiber" similarly to that described for cellulose digestibility.

The apparent digestibility of nitrogen was not significantly altered by changes in cellulose level. There was no significant

TABLE 5

The effect of varying cellulose and nitrogen levels upon average daily urinary nitrogen, nitrogen balance and lamb weight gains

RATION REGIME		AVERAGE URINARY NITROGEN [1]	NITROGEN BALANCE [1]	21-DAY WT. GAIN PER LAMB [1]	WT. OF LAMB [2]
N	Cell.				
%	%	*gm/day*	*gm/day*	*lbs.*	*lbs.*
1.65	21.4	3.0 [3]	+ 3.8 [5]	+ 1.2	85
1.65	31.4	4.5 [4]	+ 2.9 [4]	+ 2.2	84
1.65	41.6	5.0 [5]	+ 2.5	+ 2.2	85
2.05	21.4	6.0 [3]	+ 6.8 [5]	+ 0.2	89
2.05	31.4	7.9 [4]	+ 4.8 [4]	− 0.2	86
2.05	41.6	7.4	+ 4.7	− 0.1	87
2.45	21.4	7.1 [3]	+ 6.2 [5]	+ 1.5	88
2.45	31.4	9.8 [4]	+ 5.6 [4]	— 1.5	85
2.45	41.6	10.8 [5]	+ 4.7 [3]	— 2.7	86

[1] Each value is the mean of independent trials on 4 different lambs.

[2] Average weight of 4 lambs at end of 21-day trial on indicated ration.

[3] See following footnotes.

[4] Statistically larger than 3 ($P < 0.05$), within nitrogen level.

[5] Statistically larger than 4 ($P < 0.05$), within nitrogen level.

difference in digestibility between lambs consuming a constant amount of cellulose in comparison with lambs consuming variable levels of cellulose. Neither was there any significant interaction between cellulose and nitrogen level or between cellulose level, nitrogen level and constant nitrogen versus constant cellulose.

Effect of nitrogen on digestibility. Varying nitrogen levels had a quadratic effect ($P < 0.05$) upon the digestibility of organic matter, cellulose, "normal acid fiber," nitrogen-free extract, and the percentage of total digestible nutrients. The

digestibility of these proximate constituents was larger ($P < 0.05$) in rations containing 2.05% of nitrogen than in rations containing either 1.65 or 245% of nitrogen. Increases ($P < 0.005$) in nitrogen digestibility occurred between rations containing 1.65 and 2.05% of nitrogen. Nitrogen level had no significant effect upon ether extract digestibility.

Urinary nitrogen, nitrogen balance. The influence of varying levels of cellulose and nitrogen upon average daily urinary nitrogen and nitrogen balance is shown in table 5. Daily urinary nitrogen excretion increased ($P < 0.01$) with each increase in cellulose. Daily nitrogen balances decreased ($P = 0.05$) with each increase in cellulose in the ration. Maximum nitrogen retention occurred with rations containing 2.05% of nitrogen.

DISCUSSION

These results have been presented as reflecting the influence of varying cellulose and nitrogen levels; however, the 9 rations were achieved by altering a third proximate constituent, nitrogen-free extract. In the review of literature, the stimulating and the depressing influence of nitrogen-free extract have been discussed. The influence of changes in nitrogen-free extract with changes in cellulose may be removed either through partial correlation analysis or by plotting the ratio of nitrogen-free-extract intake to cellulose intake against the digestibility of cellulose, organic matter and nitrogen-free extract as shown in figure 1. Either increases in nitrogen-free extract intake or decreases in cellulose intake depressed cellulose digestibility and increased the digestibility of nitrogen-free extract and organic matter. The plot also reveals that the increased digestibility of cellulose, nitrogen-free extract, and organic matter in rations containing 2.05% of nitrogen is actually due to nitrogen level, rather than to alterations in the nitrogen-free-extract content of the ration.

A similar conclusion was obtained from partial correlations. The partial coefficients of correlation for cellulose on organic matter digestibility, with nitrogen-free extract held constant, was —0.647 and for nitrogen-free extract on organic

matter digestibility, with cellulose held constant, was + 0.672. The regression equation for these relationships was $y = 86.0 - 0.44x$ and $y = 56.4 + 0.40x'$, respectively, where y = organic matter digestibility and where x and x′ = the percentage of cellulose and nitrogen-free-extract, respectively, in the ration. The similarity, except for sign, between the coefficients of correlation and the regression coefficients (—0.44

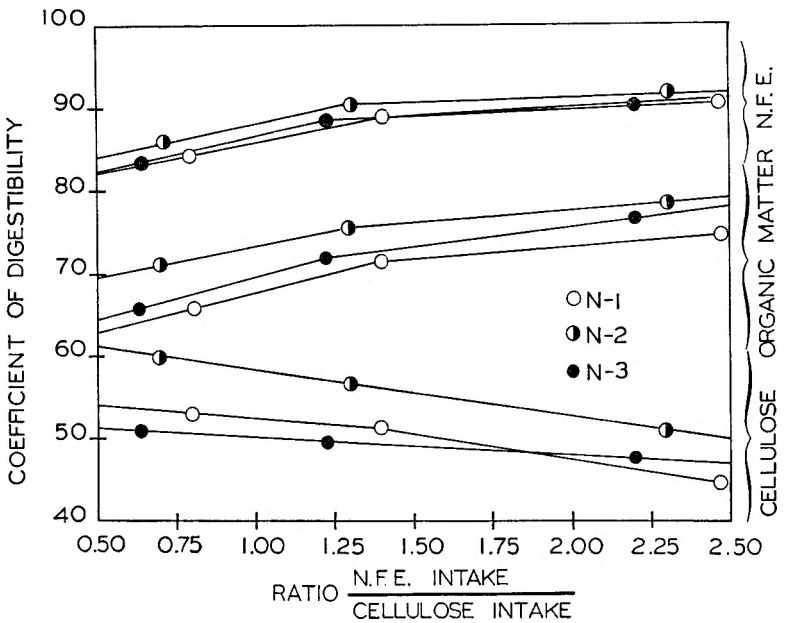

Fig. 1 Influence of the nitrogen-free extract (N.F.E.): cellulose ratio on cellulose, organic matter and N.F.E. digestibility.

and + 0.40) for the two relationships, substantiates the conclusion that the depressive effect of cellulose level on organic matter digestibility is inversely related to the levels of nitrogen-free extract.

The highly significant negative correlation ($P < 0.01$) between percentage of cellulose in the ration and organic matter digestibility reported here for semipurified rations is in accord with observations by Lancaster ('43) and Walker and Hepburn ('55) who used natural type rations. Similar rela-

tionships have been reported for crude fiber (Axelsson, '40b; McMeekan, '43; and Hallsworth, '49) and lignin (Lancaster, '43; Phillips and Loughlin, '49; Forbes and Garrigus, '50a,b). These highly significant negative correlations have suggested "envelopment" or a stable chemical association of easily digestible nutrients with poorly digested polysaccharides, thus lowering the digestibility of easily digestible nutrients either by envelopment or the formation of a highly stable chemical combination. Such conditions can easily be envisioned for natural type rations but would not be expected to occur in animals fed semipurified rations containing variable levels of relatively pure cellulose. Walker and Hepburn obtained the regression equation $y = 112.0 - 1.72x$ for the relationship between organic matter digestibility (y) and cellulose content (x) of hay rations fed to sheep. The regression coefficient reported by them (−1.72) is considerably larger than that reported in this experiment involving alterations with wood pulp (−0.44). This difference in the depressive cellulose effect upon organic matter digestibility under the two conditions employed therefore lends support to the "envelopment" concept. This suggests that before crude fiber or cellulose can be used to predict the energy value of natural feedstuffs, some estimate of the association of cellulose with other nutrients would be essential.

The depressive effect of cellulose upon the digestibility of organic matter which occurred in this experiment resulted from (1) an increasing proportion of the organic matter being composed of relatively undigestible cellulose and (2) a depressing effect of cellulose on the digestibility of the non-cellulose portion of the organic matter (protein, ether extract, and nitrogen-free extract). The digestibility of the non-cellulose organic matter [4] was calculated in order to compare the second effect singularly. The regression equation was

[4] Non-cellulose organic matter of feed and fecal material equals the sum of the percentage of nitrogen multiplied by 6.25, the percentage of nitrogen-free extract, plus the percentage of ether extract. Coefficients of digestibility were then calculated in the conventional manner.

$y = 90.2 - 0.22x$ with a highly significant coefficient of correlation ($r = -0.463$; $P < 0.01$). The regression coefficient for cellulose on organic matter digestibility (-0.44) differed significantly ($P = 0.05$) from the regression coefficient for cellulose on non-cellulose organic matter (-0.22). This difference in regression coefficients thus substantiates the influence of the second effect. Apparently it is the digestibility of the nitrogen-free-extract portion of the non-cellulose organic matter which is altered. This conclusion is similar to that reached by Lancaster ('43) who, using partial correlation analysis, concluded that the "digestibility is a function not of lignin acting as an encrusting envelope but of the whole mechanical structure of the plant." Schneider and co-workers ('51, '52) have also demonstrated that depressions in organic matter digestibility are functions not only of crude fiber but of all the proximate components.

Increased digestibility of organic matter, cellulose and nitrogen-free extract with increasing nitrogen levels conflicts with reports by Axelsson ('50) and Head ('53), who reported that protein content influenced only the digestibility of crude protein. Woods et al. ('56) reported that the addition of soybean meal to a timothy hay ration significantly increased the digestibility of dry matter, organic matter, nitrogen-free extract and energy. An examination of their basic data reveals that such increases may have been entirely the result of diluting crude fiber with nitrogen-free extract from soybean meal which is more easily digested.

The results of Head ('53) with cellulose and Woods et al. ('56) with crude fiber suggest that cellulose (or crude fiber) digestibility is not enhanced by further nitrogen additions to rations containing approximately 1% of nitrogen. The results reported here indicate that maximum cellulose digestibility is obtained with rations containing 2% of nitrogen. Depressed digestibility of cellulose, organic matter and nitrogen-free extract by further nitrogen additions to rations containing 2% of nitrogen is surprising and without explanation at the present time.

Watson et al. ('47) concluded that nutritive ratios between approximately 1 : 2 and 1 : 9 had no effect upon the digestibility of any proximate component of mixed rations for steers. The data reported here do not indicate any consistent trend between nutritive ratio and maximum digestibility. For instance, the 4 highest TDN values occurred with a nutritive ratio of 4.9, 6.0, 6.3 and 10.1.

The significantly increased urinary nitrogen excretion with increases in ration cellulose suggests that protein was being catabolized to compensate for reduced total digestible nutrient intake. This same trend is also reflected in daily nitrogen balances which decrease with increasing cellulose levels. The depressed weight gains of lambs fed progressively higher nitrogen rations might be a reflection of the high specific dynamic action of protein metabolism. Crampton ('39) and Crampton and Forshaw ('40) reported that increasing the cellulose level, although tending to depress weight gains, had no significant effect on the weight gains of rabbits and cattle respectively. Cellulose level, as reported here, seemed to be without consistent effect on weight gains. Since it is apparent that increasing cellulose level increased protein catabolism, little significance can be attached to 21-day weight gains reported here.

SUMMARY

Lambs were fed semipurified rations containing one of three levels of cellulose (21.4, 31.4 or 41.6%) and one of three nitrogen levels (1.65, 2.05 or 2.45%). Cellulose and nitrogen levels were varied by substituting a purified source of cellulose and protein for cornstarch. Increasing the cellulose levels significantly and linearly increased cellulose and ether extract digestibility; decreased the digestibility of organic matter, nitrogen-free extract and reduced percentage of total digestible nutrients, and was without significant effect upon the apparent nitrogen digestibility. Increasing nitrogen levels significantly and quadratically influenced the digestibility of organic matter, cellulose, nitrogen-free extract,

nitrogen and the percentage of total digestible nutrients. The digestibility of nitrogen was not further increased by increasing ration nitrogen from 2.05 to 2.45%; the digestibility of the other nutrients in rations containing 2% of nitrogen was significantly higher than in rations containing either 1.65 or 2.45% of nitrogen.

The influence of cellulose upon the digestibility of organic matter in semipurified rations was shown to be due both to its diluting effect (upon easily digestible components) and to its depressive effect upon the digestibility of non-cellulose organic matter. This depressive effect is approximately one-fourth that reported for natural type rations. This is interpreted as supporting the "envelopment" concept of depressed nutrient digestibilities with increasing cellulose concentration.

Increased cellulose levels significantly increased daily urinary nitrogen and decreased daily nitrogen balances.

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EFFECTS OF THE PREVENTION OF COPROPHAGY IN THE RAT

III. DIGESTIBILITY OF PROTEIN AND FAT

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With the development of a technique for the prevention of coprophagy in the rat (Barnes et al., '57) it has become possible to determine the influence of fecal recycling upon the digestibility of the major foodstuffs. Since it has been estimated that the rat consumes about 50% of its feces, even though maintained in wire bottom cages, it is logical to assume that poorly digestible protein or fat might be more completely absorbed from the gastrointestinal tract if permitted to recycle as a consequence of coprophagy. If this were the case, the classical digestibility information that has been obtained with the laboratory rat would have to be re-interpreted. In another species, the rabbit, Thacker and Brandt ('55) have shown that coprophagy may increase remarkably the digestibility of protein and to a less dramatic but measurable extent, increase the digestibility of fat.

EXPERIMENTAL

Two protein sources and one fat source were chosen for study. Casein, as an example of a very highly digestible protein, was compared with the mixed proteins of a commercial dog food [1] as an example of protein of lower digestibility. The fat employed in the purified diets was a hydrogenated vegetable shortening [2] that was known to be of intermediate digestibility.

[1] G. L. F. Big Red Dog Food.
[2] Procter and Gamble "Primex."

Young adult male rats weighing in the approximate range 200 to 250 gm were used in all experiments. The composition of the normal-protein, purified diet is given in table 1. The low-protein and protein-free diets were made by reducing the amount of casein and replacing it by an equivalent weight of cerelose. Ten rats were used in each group and coprophagy prevention was accomplished by the technique previously described by Barnes et al. ('57). Feces were collected daily from the collection cups or from absorbent paper under the wire screen-bottom cages. Fecal collections were pooled for each group each day. Four daily collections were kept separate.

TABLE 1

Composition of "normal protein" diet

MAJOR COMPONENTS	
	gm
Casein [1]	25.0
Cerelose	53.0
Primex [2]	15.0
Salts [3]	4.0
Choline dihydrogen citrate	0.3
B vitamins in sucrose	2.0
Fat-soluble vitamins in corn oil	1.0
	100.3

B VITAMINS IN 2.0 GM SUCROSE		FAT-SOLUBLE VITAMINS IN 1.0 GM CORN OIL	
	mg		*mg*
Thiamine HCl	0.40	Vitamin A acetate	0.31
Riboflavin	0.80	Vitamin D (calciferol)	0.0045
Pyridoxine HCl	0.40	Alpha tocopherol	5.00
Ca pantothenate	4.00		
Niacin	4.00		
Inositol	20.00		
Biotin	0.02		
Folic acid	0.20		
Vitamin B_{12}	0.03		
Menadione	1.00		

[1] Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Procter and Gamble, Cincinnati, Ohio.

[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14:* 273 (1937).

Feces were weighed, dried in an oven at 100°C for 48 hours, and then weighed again for a calculation of moisture content. Dried feces were ground and sampled for micro Kjeldahl determination of nitrogen and gravimetric determination of fat. Fecal samples were acidified with concentrated HCl, extracted 4 times with 25 ml of boiling 1:1 absolute ethanol and ether. The pooled extracts were reduced to small volume by evaporation under reduced pressure and extracted with petroleum ether (30° to 60°C). The fecal fat was weighed after complete removal of the solvent from petroleum ether extract.

In all experiments rats were maintained on the experimental diets for a minimum of 10 days to two weeks before starting fecal collections. Food was allowed ad libitum and consumption was measured only during the 4 days of fecal collections.

RESULTS

An analysis of the food intake, fecal output and moisture content of the feces is given in table 2. Consistent with previously reported findings, the food intake and fecal output of the rats that had been prevented from eating their feces were both smaller than was found in normal animals. This relationship held true regardless of the diet group with the exception of the rats on a protein-free diet. In this instance both food consumption and fecal output were greater in the group in which coprophagy was prevented. The water content of the feces that had been collected in the plastic cups was approximately twice the amount in the feces that were collected from under the wire screen bottoms of the cages. Absorption of water by the paper, and evaporation, probably explain this rather large difference.

The nitrogen of the feces from rats that were receiving a "protein-free" diet was used as a measure of "metabolic fecal nitrogen." When expressed as grams of fecal nitrogen per 100 gm of food ingested, as recommended by Schneider ('35), the value was 0.085 for the normal rats and 0.097 for those prevented from eating their feces. In table 3 it will be noted that when the protein absorption figures are corrected

TABLE 2

Food intake and fecal excretion

GROUP DESCRIPTION	NO. OF RATS [1]	DIET PROTEIN N × 6.25	AVERAGE BODY WEIGHT	FOOD INTAKE PER RAT PER DAY	FECES PER RAT PER DAY (DRY WT.)	WATER CONTENT OF FECES
		%	*gm*	*gm*	*gm*	%
Normal protein control	20	20.9	263	18.8	0.95	14.3
Normal protein — feces cups	20	20.9	236	17.0	0.82	29.4
Low protein — control	20	10.4	196	18.6	1.06	10.4
Low protein — feces cups	20	10.4	184	16.4	0.92	29.5
Protein free — control	10	0.12	102	9.1	0.46	14.3
Protein free — feces cups	10	0.12	103	9.5	0.48	31.0
Commercial chow — control	10	26.2	239	21.9	5.33	34.7
Commercial chow — feces cups	10	26.2	213	21.4	4.85	64.4

[1] Ten rats per group per experiment; 20 rats indicates two separate experiments.

by these amounts, the "true" digestibility of dietary protein was the same in both groups of rats. Equal digestibilities were found for casein, as an example of a well-digested protein (approximately 99%), and the protein of a commercial dog chow, as an example of a poorly-digested protein (approximately 79%).

No attempt was made to measure the "endogenous" fecal lipids. However, as seen in table 4, the apparent digestibilities of fat in both normal and coprophagy-prevented rats were so nearly identical that it is doubtful if any major difference in endogenous lipid could have existed. The lowered digestibility of fat that is associated with a decreased protein content of the diet was noted in confirmation of earlier reports (Barnes et al., '44). The fat of the dog chow was supposedly largely a commercial tallow and appeared to have a slightly lower digestibility than the hydrogenated shortening that was used in the purified diets.

DISCUSSION

Although no measurable changes in the digestibility of protein or fat were observed in these studies, qualitative differences of importance in the normal and coprophagy-prevented rat may exist. For example, Norcia and Lundberg ('54) have shown that rat feces contain a small but fairly constant amount of linoleic and possibly other essential fatty acids. These authors have suggested that this endogenous fat probably results from microbiological synthesis in the large intestine. If one were attempting to develop an essential fatty acid deficiency in the rat, the consumption of a large portion of feces might supply a small, but important, part of the animal's requirement of these acids. The lack of any gross difference in digestibility does not necessarily rule out the possibility of a qualitatively important consumption of fatty acids as a consequence of coprophagy. The observation that undigestible fat does not become available upon reconsumption is difficult to understand.

TABLE 3

Protein (nitrogen) digestibility as affected by coprophagy

GROUP DESCRIPTION	INTAKE		OUTPUT		DIGESTIBILITY		
	Total food intake per rat per day	N per 100 gm food	Total N in feces per rat per day	N excreted per 100 gm food ingested	"Apparent" digestibility	Metabolic fecal N	"True" Digestibility
	gm	*gm*	*gm*	*gm*	%	*gm*	%
Normal protein — control	18.8	3.348	0.025	0.132	96.0	0.085	98.6
Normal protein — feces cups	17.0	3.348	0.021	0.125	96.2	0.097	99.1
Low protein — control	18.6	1.665	0.018	0.099	94.0	0.085	99.1
Low protein — feces cups	16.4	1.665	0.018	0.109	93.4	0.097	99.2
Protein free — control	9.1	0.019	0.008	0.085	—	0.085	—
Protein free — feces cups	9.5	0.019	0.009	0.097	—	0.097	—
Commercial chow — control	21.9	4.194	0.221	1.009	75.9	0.085	78.0
Commercial chow — feces cups	21.4	4.194	0.203	0.948	77.4	0.097	79.7

TABLE 4

Fat digestibility as affected by coprophagy

GROUP DESCRIPTION	INTAKE			OUTPUT	
	Diet protein	Diet fat	Fat intake per rat per day	Fat in feces per rat per day	"Apparent" digestibility
	%	%	*gm*	*gm*	%
Normal protein — control	21.0	14.70	2.706	0.134	94.9
Normal protein — feces cups	21.0	14.70	2.594	0.116	95.6
Low protein — control	10.4	15.18	2.945	0.260	91.1
Low protein — feces cups	10.4	15.18	2.572	0.204	92.0
Commercial chow — control	26.2	7.03	1.543	0.102	93.4
Commercial chow — feces cups	26.2	7.03	1.506	0.098	93.6

Protein digestibility was also uninfluenced by the prevention of coprophagy. This is in direct contrast to results obtained in the rabbit by Thacker and Brandt ('55) where prevention of coprophagy markedly decreased protein digestibility. On the other hand, the rabbit may have a relatively large "metabolic" fecal nitrogen. Nitrogen derived from intestinal secretions and desquamation might well be digestible if recycled through the small intestine. The small difference in "metabolic" fecal nitrogen between the normal and coprophagy-prevented rat is in line with this suggestion. Protein that is not digested as evidenced by its presence in feces is truly undigestible even though subjected to several passages through the entire intestinal tract.

An important by-product of this study is the assurance that the extensive fat and protein digestibility studies that have been conducted in the rat probably are not affected quantitatively by the practice of copraphagy.

SUMMARY

The practice of coprophagy does not influence to any measurable extent the digestibility of either fat or protein in the rat.

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THE RETENTION OF FLUORIDE BY THE SKELETON, LIVER, HEART AND KIDNEY AS A FUNCTION OF DIETARY FAT INTAKE IN THE RAT [1]

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Miller and Phillips ('55) have presented data indicating an enhancement of fluoride toxicity in rats which received a diet containing 15 or 20% of cottonseed oil when compared to a similar group of animals receiving a diet in which there was only 5% of cottonseed oil. The criterion by which fluoride toxicity was evaluated in these studies was the growth-retarding effect of the different diets, all of which contained 0.1% of sodium fluoride, and the increased deposition of fluoride in the femora. As a result of these studies it became of interest to determine what effect the feeding of diets containing a number of different fats would have on the retention of fluoride in the skeleton as well as in various soft tissues of the rat. Previous studies by Buttner and Muhler ('57) have shown a retention of fluoride in the heart and kidney when 2 mg of fluoride daily are ingested by rats, and that dietary fat seems to enhance the retention of fluoride in certain soft tissues. Muhler ('58) has also shown that the presence of vitamin C increases fluoride retention both in the skeleton and soft tissues of the guinea pig. The mechanism by which this increased retention in the soft tissues occurs is not known,

[1] This study was supported in part by a grant from the Procter and Gamble Company, Cincinnati, Ohio.

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and much additional work is needed in order to determine what factors govern fluoride deposition in the soft tissues and what physiological role, if any, the presence of fluoride has in these tissues.

EXPERIMENTAL

A total of 150 weanling male Sprague-Dawley strain rats were divided equally into 5 series. Series I was divided into three groups, all of which received a stock sucrose diet containing 5% cottonseed oil. Group 1 animals received no added fluorine, group 2 received sodium fluoride at a concentration of 30 μg F/ml in the drinking water, and group 3 received 2 mg of fluoride daily (as sodium fluoride) by stomach tube. Each of the 4 remaining series was divided similarly into three groups and received the same amount of fluorine as series I animals either in the drinking water or by stomach tube, but the type and level of dietary fat was changed. The animals in series II received cottonseed oil, those in series III corn oil, series IV hydrogenated vegetable oil,[3] and series V lard. All of the fat was added at a level of 20%. Animals in groups 1 and 3 of each series received a fluoride-low (F = 0.05 μg/ml) drinking water ad libitum. The composition of the experimental diets, which were also available ad libitum, are found in summary form in table 1.

All of the animals were housed individually in raised screen cages in an air-conditioned room and were weighed twice each week throughout the experimental period which lasted 10 weeks. At the termination of the experiment the animals were sacrificed by ether, and fluoride in the femora, whole carcass, liver, kidney, and heart was determined by methods previously described (Weddle and Muhler, '54).

RESULTS AND DISCUSSION

As judged by body weight gains, the effect of the different fats or the presence of fluorides in any of the diets, is not

[3] Crisco.

TABLE 1

Composition of experimental diets

COMPONENT OF DIET	ANIMAL SERIES														
	I			II			III			IV			V		
	Group			Group			Group			Group			Group		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Sucrose	60	60	60	45	45	45	45	45	45	45	45	45	45	45	45
Vitamin test casein	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Cottonseed oil [1]	5	5	5	20	20	20	—	—	—	—	—	—	—	—	—
Corn oil	—	—	—	—	—	—	20	20	20	—	—	—	—	—	—
Hydrogenated vegetable oil [2]	—	—	—	—	—	—	—	—	—	20	20	20	—	—	—
Lard	—	—	—	—	—	—	—	—	—	—	—	—	20	20	20
Salt mixture [3]	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Vitamin mixture [3]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Oleum percomorphum [4]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sodium fluoride [5]	—	*	**	—	*	**	—	*	**	—	*	**	—	*	**

[1] Wesson.

[2] Crisco.

[3] Muhler ('54).

[4] Fifteen drops per kilo of diet.

[5] * — 30 μg F/ml added to drinking water.

** = 2.0 mg F per day by stomach tube.

associated with toxic effects. In all cases the animals which received fluoride weighed as much if not more than their respective controls not receiving fluorides. Similarly the different fats did not produce significant differences in weight gain. Thus, the enhancement of toxicity as evaluated by failure to gain weight normally as reported by Miller and Phillips ('55) in which 15 or 20% cottonseed oil had been used was not confirmed. However, since the type of diet used by Miller and Phillips was considerably different from that used in these studies, the total amount of fluoride ingested by their animals was in all probability considerably greater than received by our animals, thus making invalid any growth comparisons between the two studies. Their diet contained 0.1% of sodium fluoride and if their animals ate 20 gm of food per rat per day, approximately 10 mg of fluoride per day per rat was ingested, a value 5 times in excess of the highest amount ingested by any of our animals in these studies.

The fluoride data of the skeleton and soft tissues obtained in this study are presented in tables 2 through 4. Table 2 shows the results of fluoride retention in the femur as a function of both dietary fat intake and the level of fluoride received by the rats. Neither the amount nor type of fat in the diet is associated with any significant difference in fluoride storage in the control animals. The total amount of fluoride was almost identical in all instances, but the concentration varied considerably. There is a tendency for a higher concentration of fluoride in the rats receiving 20% of cottonseed oil and a lower amount in those animals receiving 20% of lard when compared to those animals receiving 5% of cottonseed oil, although these differences have only borderline significance ($p = 0.06$). When one examines the data obtained from the animals which received 30 μg F/ml in their drinking water the same relative increase as noted in the control group with 20% of cottonseed oil is found, although this difference is significantly at the 0.01 level of confidence. With the use of all other fats there is less fluoride in the femur in comparision with the amounts deposited in the animals

TABLE 2

Effect of fat on the fluorine content of the ash of femora in rats

DIETARY FAT	CONTROL		30 P.P.M. F IN THE DRINKING WATER		2 MG F DAILY BY STOMACH TUBE	
	Total F	Conc. F	Total F	Conc. F	Total F	Conc. F
%	*mg*	*p.p.m.*	*mg*	*p.p.m.*	*mg*	*p.p.m.*
Cottonseed oil, 5	0.03 ± 0.003 [1]	98 ± 8 [1]	0.61 ± 0.05 [1]	2180 ± 170 [1]	1.45 ± 0.07 [1]	5620 ± 370 [1]
Cottonseed oil, 20	0.03 ± 0.003	125 ± 10	0.74 ± 0.08	2610 ± 250	1.72 ± 0.23	6440 ± 470
Corn oil, 20	0.03 ± 0.002	82 ± 5	0.51 ± 0.05	2100 ± 140	1.83 ± 0.15	6460 ± 270
Crisco, 20	0.03 ± 0.003	100 ± 6	0.43 ± 0.03	1910 ± 280	1.80 ± 0.05	7950 ± 390
Lard, 20	0.02 ± 0.002	80 ± 9	0.56 ± 0.03	2110 ± 80	1.75 ± 0.08	7310 ± 290

[1] Standard deviation.

TABLE 3

Effect of fat on the fluorine content of the ash of the carcass of rats

DIETARY FAT	CONTROL		30 P.P.M. F IN THE DRINKING WATER		2 MG F DAILY BY STOMACH TUBE	
	Total F	Conc. F	Total F	Conc. F	Total F	Conc. F
%	*mg*	*p.p.m.*	*mg*	*p.p.m.*	*mg*	*p.p.m.*
Cottonseed oil, 5	0.8 ± 0.1 [1]	100 ± 20 [1]	12.3 ± 1.2 [1]	1500 ± 200 [1]	27 ± 2 [1]	3600 ± 400 [1]
Cottonseed oil, 20	0.8 ± 0.1	100 ± 10	15.4 ± 0.7	1900 ± 70	29 ± 3	3900 ± 400
Corn oil, 20	0.7 ± 0.1	80 ± 10	13.4 ± 0.8	1700 ± 80	28 ± 2	3300 ± 400
Crisco, 20	1.2 ± 0.3	140 ± 30	11.6 ± 0.9	1700 ± 110	32 ± 2	4900 ± 300
Lard, 20	0.7 ± 0.1	100 ± 10	9.7 ± 1.0	1300 ± 120	50 ± 10	7000 ± 1500

[1] Standard deviation.

receiving fat at the 5% level. In the animals receiving 2 mg of fluoride daily there is again a tendency for more fluoride in the 20% cottonseed oil group but the difference is not highly significant ($p = 0.06$). The ingestion of corn oil, Crisco, or lard results in significantly higher ($p = 0.01$) fluoride retention values, however, when compared to the 5% fat group. It is evident from these data that the level of fluoride ingested is one of the predominant factors in determining the enhancement of fluoride toxicity by dietary fats.

The fluoride retention in the whole carcass is seen in table 3. The relative data from the animals receiving no extra dietary fluoride are quite similar in the whole carcass to those found in the femur in that no significant differences were seen when any of the different fats at the 20% level were used. When 30 μg F/ml was fed there was a significant ($p = 0.03$) increase in total fluoride when 20% cottonseed oil was fed and a significant reduction ($p = 0.02$) when lard was the source of fat when compared to the 5% cottonseed oil group. The differences are similar in magnitude when the concentration is used for evaluation, although in this instance only the 20% cottonseed oil is significantly different. When 2 mg of fluoride is ingested daily all of the animals fed fats at the 20% level have more total fluoride in the femora, but only in those animals receiving Crisco and lard are the values significantly different ($p = 0.01$) from those of the 5% cottonseed animals.

Much additional information is needed in order to evaluate properly the reasons for differences in fluoride retention when the whole carcass or femur are used for comparative purposes. One such possible explanation could be the amount of fluoride deposited in the various soft tissues when the whole carcass is used. Table 4 shows the effect of both fluoride and fat intake on fluoride retention in the liver, heart and kidney. The administration of 2 mg F per day results in more fluoride retention in the liver, heart and kidney, regardless of the type of fat ingested, in all instances except in the heart of the animals receiving 5% cottonseed oil. In all other instances

TABLE 4

Effect of fat on the fluorine content of liver, heart and kidney of the rat [1]

DIET	LIVER		HEART		KIDNEY	
	Total F	Conc. F	Total F	Conc. F	Total F	Conc. F
	μg	*p.p.m.*	*μg*	*p.p.m.*	*μg*	*p.p.m.*
5% Cottonseed oil	0.0	0.0	1.7	3.4	0.7	0.5
5% Cottonseed oil + 30 μg F/ml	0.0	0.0	1.0	1.4	1.1	0.7
5% Cottonseed oil + 2 mg F/da	2.0	0.5	1.1	1.8	1.0	0.8
20% Cottonseed oil	1.1	0.2	0.8	1.0	1.7	0.9
20% Cottonseed oil + 30 μg F/ml	3.3	0.9	0.6	0.7	2.1	1.4
20% Cottonseed oil + 2 mg F/da	4.2	0.9	1.0	1.6	2.1	1.4
20% Corn oil	1.0	0.2	0.0	0.0	0.3	0.2
20% Corn oil + 30 μg F/ml	5.1	1.6	0.3	0.4	0.7	0.6
20% Corn oil + 2 mg F/da	3.1	0.8	1.2	2.8	2.2	3.0
20% Crisco	1.9	0.8	0.9	1.5	0.4	0.2
20% Crisco + 30 μg F/ml	1.8	0.5	0.9	1.8	0.7	0.6
20% Crisco + 2 mg F/da	3.3	1.0	1.3	1.8	2.4	1.6
20% Lard	3.0	0.8	1.3	1.7	1.0	0.6
20% Lard + 30 μg F/ml	5.5	1.0	1.0	1.4	0.5	0.3
20% Lard + 2 mg F/da	6.6	1.8	1.9	5.8	3.4	2.2

[1] Each value represents the mean value obtained by pooling for analysis three livers, 6 hearts or 6 kidneys. This was necessary since such microquantities of fluorine are found in the soft tissues that accurate individual organ analysis is not possible. Such a procedure eliminates the possibility of calculating a value for the standard deviation.

the administration of 2 mg F per day results in more fluoride in each of these tissues than in the respective controls not receiving added dietary fluoride. Similar tendencies are evident also in the animals receiving 30 μg F/ml in the drinking water, although in these animals, the differences are not as great. The greatest numerical increase in fluoride retention occurs in the liver of the animals receiving 20% of lard, although in general, all the liver values are appreciably higher than those found in the heart or kidney. The physiological significance of this increased retention of fluoride in the liver, kidney and heart is not known.

SUMMARY

The effect of feeding different dietary fats at a 5 and 20% level in the presence or absence of fluoride has been studied in order to determine if either the amount or type of fat is associated with increased retention of fluoride in the skeleton, liver, heart and kidney in the rat. Increasing the dietary fat from 5 to 20% results in more fluoride retention in the whole carcass, femur and soft tissues when 2 mg of fluoride is fed daily. Neither the dietary fats nor the presence of the fluoride at the levels used in these studies was associated with a decrease in body weight gain.

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STUDIES ON ZINC DEFICIENCY IN THE CHICK

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Little information is available concerning the requirement of various animal species for zinc. Growing rats fed purified diets nearly devoid of zinc, develop symptoms of zinc deficiency characterized by retarded growth and poor hair development (Todd et al., '34; Stirn et al., '35). Recent investigations (Tucker and Salmon, '55; Lewis et al., '56; Luecke et al., '56 and others) indicate that a dermatitis, prevented or cured by zinc salts, may be produced in swine fed practical-type diets which contain appreciable quantities of zinc. O'Dell and Savage ('57) have briefly reported that added zinc stimulated the growth of chicks fed a semipurified soybean protein [1] diet containing approximately 50 p.p.m. of zinc. The present report shows that zinc deficiency in the chick, characterized by retarded growth and abnormal bone formation, may be produced with semipurified diets containing 30 p.p.m. of zinc. The dietary requirement of the chick for zinc was found to be markedly influenced by the calcium content of the diet. Evidence was obtained suggesting that the zinc in a diet containing soybean protein may be less available to the chick than that in a similar diet containing casein and gelatin.

EXPERIMENTAL

The composition of the basal diets used in the studies reported herein is shown in table 1. The soybean protein [1]

[1] Drackett Assay Protein C–1, The Drackett Products Company, Cincinnati, Ohio.

in diet A was used as received from the manufacturer. The protein in diet B was supplied by a combination of vitamin-test casein and gelatin. The mineral mixture in both diets was made from reagent grade compounds, and was identical to that used by Morrison et al. ('56), except that sodium bromide was added to supply 5 p.p.m. of bromine to the diet.

TABLE 1

Composition of basal diets

INGREDIENT	DIET A	DIET B
Sucrose, gm	61.09	59.26
Soybean protein, gm [1]	25.47	—
Purified casein, gm [2]	—	18.00
Gelatin, gm	—	10.00
DL-Methionine, gm	0.70	0.30
Glycine, gm	0.30	—
Non-nutritive fiber, gm [3]	3.00	3.00
Hydrogenated fat, gm [4]	3.00	3.00
Mineral mixture, gm [5]	5.43	5.43
Vitamin mixture in sucrose, gm [6]	0.81	0.81
Cod liver oil, gm [7]	0.20	0.20

[1] Drackett Assay Protein C-1, The Drackett Products Company, Cincinnati, Ohio.

[2] Labco Vitamin Test Casein, The Borden Company, New York, New York.

[3] General Bioehemicals Incorporated, Chagrin Falls, Ohio.

[4] Primex, Procter and Gamble Company, Cincinnati, Ohio.

[5] Same as that of Morrison et al. ('56) except that 0.64 mg NaBr was added per 100 gm of diet.

[6] Vitamins — choline bitartrate, 0.31 gm; inositol, 25.0 mg; niacin, 5.0 mg; calcium pantothenate, 2.0 mg; folic acid, 0.4 mg; menadione, 0.05 mg; pyridoxine HCl, 0.45 mg; riboflavin, 1.0 mg; thiamine·HCl, 1.0 mg; biotin, 20.0 μg; vitamin B_{12}, 0.5 μg; a-tocopheryl acetate, 2 mg per 100 gm of diet.

[7] Mead Johnson and Company. The material contained 1850 units of vitamin A and 175 units of vitamin D per gm.

Bromine was added to the diets because of the reports of Bosshardt et al. ('56) and Huff et al. ('56) that bromine stimulated the growth of mice and chicks fed purified diets. The mineral mixture contained molybdenum, which has been reported by Reid et al. ('56) to stimulate chick growth under certain conditions. The amount of mineral mixture used added 1.23% of calcium and 0.69% of phosphorus to the diets. Both basal diets contained approximately 30 p.p.m. of zinc, as

determined by the method for zinc analysis in foods of the Association of Official Agricultural Chemists ('55), of which 4.8 p.p.m. was supplied by the mineral mixture. In experiments in which minerals were aded to the basal diets, additions were made at the expense of sucrose.

Male White Plymouth Rock chicks [2] obtained from a local commercial hatchery, were used in the experiments. On receipt from the hatchery, the chicks were identified with numbered wing bands and placed in electrically heated galvanized metal chick Brood-Units with raised screen floors, kept in an air-conditioned room maintained at 75 to 77°F. Feed was supplied in galvanized metal troughs. In experiment 1, tap water was supplied in galvanized pans, but in subsequent experiments distilled water was supplied in glass jars. Substitution of distilled water for tap water appeared to have no effect on the response of chicks to zinc supplementation. The chicks received the basal diets ad libitum for the first 12 days of life, at which time they were separated into the various experimental groups on the basis of 11-day weights and 5- to 11-day weight gains by a modification of the selection method of McKittrick ('47). The animals then received the experimental diets ad libitum from 12 to 26 days of age. They were individually weighed at weekly intervals and records were kept of the amount of feed consumed per pen of chicks during the experiment.

In experiment 1, duplicate groups of 10 chicks each received the soybean protein basal diet (diet A) or diet A supplemented with 5, 25 or 100 p.p.m. of zinc. In this and subsequent experiments, zinc was added as reagent grade zinc chloride.

Experiment 2 was conducted to determine if the tissues laid down by chicks receiving diet A were of similar composition to those of chicks fed diet A supplemented with 25 p.p.m. of zinc. At the end of the growth period, the animals were fasted for 18 hours and then sacrificed by intraperitoneal injection of Nembutal [3] solution. The liver and heart weights

[2] Arbor Acres strain.

[3] Abbott.

of each chick were recorded and the livers taken for analysis of dry matter, ether-extract and protein (N × 6.25). The dry matter and ether-extract were determined by the method of Sarett and Jandorf ('47) and the nitrogen content of the dried fat-free liver by the Kjeldahl method. Protein and ether-extract levels were calculated on a fresh weight basis. The carcass of each animal (minus the liver and intestinal tract) was placed in a tared Mason jar, autoclaved at 15 pounds steam pressure for 4 hours, homogenized in a Waring Blendor and analyzed for dry matter, ether-extract, protein (N × 6.25) and ash by previously described methods (Sarett and Jandorf, '47).

Since previous studies (Tucker and Salmon, '55; Lewis et al., '56; Luecke et al., '57; Conrad and Beeson, '57 and others) indicated that in swine a relationship exists between the level of dietary calcium and the response to zinc, the effect of addition of calcium to diet A on the response of chicks to added zinc was tested in experiment 3. Calcium was added as reagent grade calcium carbonate. At the end of the experiment, the birds in each pen were listed according to weight and alternate birds were sacrificed by chloroform anesthesia. The right tibiotarsae were removed for determination of bone ash by the method of the Association of Official Agricultural Chemists ('55) and the length and width of the tibiotarsae of the chicks from some groups were measured with a micrometer.

In experiment 4, the effect of addition of zinc to the casein-gelatin diet (diet B) was studied, in the presence and absence of added calcium.

In each of the experiments, the standard deviations of the weight gains were calculated and the data were subjected to appropriate statistical analysis by the methods outlined by Snedecor ('55).

RESULTS AND DISCUSSION

The average weight gains and efficiencies of feed utilization of the chicks which received the various diets in experiment

1 are shown in table 2. During the 14-day experimental period (days 12 to 26) the animals which received diet A gained an average of 195 gm, whereas those which received diet A supplemented with 5, 25, and 100 p.p.m. of zinc gained 234, 244 and 231 gm respectively. Each of the latter weight gains was significantly greater than that of the chicks which received diet A ($p < 0.01$ by analysis of variance). No significant differences between the growth responses obtained from the three levels of zinc were observed. Efficiencies of feed utilization were increased more than 10% by addition of zinc to diet A. These increases are believed to be significant, since the average values for replicate groups were in close agreement, although the data are not amenable to rigid statistical treatment.

The observation that addition of as little as 5 p.p.m. of zinc to diet A significantly stimulated weight gains was somewhat unexpected, since diet A contained 30 p.p.m. of zinc. Further, the chicks used were the progeny of hens fed commercial diets and were housed in galvanized brooders. The results suggest that part of the zinc in diet A may be unavailable to the chick, or that the zinc requirement of the chicks used was greater than 30 p.p.m. of diet.

The results of experiment 2, conducted to determine the effects of the addition of zinc to diet A on organ weights and liver and carcass composition, are summarized in table 3. The stimulatory effect of zinc on weight gains was again noted. The losses in weight during the 18-hour fast before the animals were sacrificed were similar in both groups. The heart and liver weights, expressed as percentage of body weight, were similar in animals fed diet A and those fed diet A supplemented with 25 p.p.m. of zinc. The levels of dry matter, fat and protein in the livers of the animals fed diet A (25.2, 4.1 and 23.1% respectively) were similar to those in the livers of animals fed diet A supplemented with zinc (25.1, 3.9 and 23.3%, respectively). The data on carcass analysis show that addition of zinc to diet A had no significant influence on the composition of the tissues laid down. The percentages of dry

TABLE 2

Effect of addition of graded amounts of zinc to diet A on chick growth [1]
(Experiment 1)

DIET NO. AND DESCRIPTION	AVERAGE WEIGHT AT			AVERAGE GAIN 12–26 Days	GAIN OVER BASAL	FEED EFFICIENCY [2] 12–26 Days
	12 Days (Initial)	19 Days	26 Days			
	gm	*gm*	*gm*	*gm*	%	
1. Basal	126	217	329	195 ± 27.9	—	0.59
(diet A)	126	205	313			
2. + 5 p.p.m. Zn	123	226	354	234 ± 27.2	20.0	0.67
	127	234	364			
3. + 25 p.p.m. Zn	127	232	369	244 ± 31.7	25.1	0.68
	126	231	373			
4. + 100 p.p.m. Zn	125	224	355	231 ± 25.2	18.5	0.64
	126	226	356			

[1] Figures shown for average weights represent mean of each pen of 10 chicks. Those for average gains represent the mean of the two replicates, with standard deviations.

[2] Average grams gain per gram of feed consumed. Figures shown represent the mean of the two replicates.

matter, fat, ash and protein in the carcasses of chicks fed diet A were found to be 33.8, 9.6, 3.5 and 20.6, respectively. The corresponding values for the chicks which received supplemental zinc were 33.7, 10.5, 3.2 and 20.5%, respectively.

The data on weight gains and efficiencies of feed utilization obtained in experiment 3, conducted to study interrelationships between the zinc and calcium levels in diet A, are summarized in table 4. The addition of 0.5 or 1.0% of calcium to diet A decreased weight gains significantly ($p < 0.01$ by variance analysis) from 188 gm to 161 gm and 152 gm, re-

TABLE 3

Effect of addition of zinc to soybean protein diet (diet A) on weight gains, fasting weight losses, heart and liver weights and liver and carcass composition of chicks[1]
(Experiment 2)

MEASUREMENT	DIET A	DIET A + 25 P.P.M. ZINC
Average weight gain, gm	184 ± 27.5	246 ± 25.2
Fasting weight loss, % in 18 hours	6.1	5.1
Heart weight		
Grams	1.98	2.27
% Body weight	0.68 ± 0.09	0.64 ± 0.10
Liver weight		
Grams	8.8	10.1
% Body weight	3.0 ± 0.4	2.9 ± 0.4
Liver composition		
Dry matter, %	25.2 ± 1.7	25.1 ± 1.7
Fat (ether-extract), %	4.1 ± 0.3	3.9 ± 0.3
Protein (N × 6.25), %	23.1 ± 1.3	23.3 ± 1.3
Carcass composition		
Dry matter, %	33.8 ± 1.5	33.7 ± 1.7
Fat (ether-extract), %	9.6 ± 2.0	10.5 ± 1.1
Ash, %	3.5 ± 0.2	3.2 ± 0.2
Protein (N × 6.25), %	20.6 ± 0.3	20.5 ± 0.3

[1] Two lots of 10 chicks were fed each diet.
Some values shown with standard deviations.

TABLE 4

Studies on interrelationships between zinc and calcium in chick growth [1]

(Experiment 3)

DIET NO. AND DESCRIPTION	AVERAGE WEIGHT AT			AVERAGE GAIN 12–26 DAYS	FEED EFFICIENCY [2] 12–26 DAYS
	12 Days (Initial)	19 Days	26 Days		
	gm	*gm*	*gm*	*gm*	
1. Basal (diet A)	123	209	315	188 ± 36.6	0.57
	121	200	305		
2. + 0.5% Ca	127	201	293	161 ± 27.7	0.55
	123	194	279		
3. + 1.0% Ca	125	194	276	152 ± 29.2	0.53
	123	192	276		
4. + 5 p.p.m. Zn	126	224	345	218 ± 25.0	0.60
	124	216	341		
5. + 0.5% Ca, 5 p.p.m. Zn	126	211	316	194 ± 27.3	0.59
	125	216	324		
6. + 1.0% Ca, 5 p.p.m. Zn	123	195	293	172 ± 28.9	0.55
	125	209	299		
7. + 25 p.p.m. Zn	125	221	350	224 ± 30.6	0.61
	124	223	347		
8. + 0.5% Ca, 25 p.p.m. Zn	124	230	364	240 ± 28.2	0.60
	123	223	364		
9. + 1.0% Ca, 25 p.p.m. Zn	122	221	351	236 ± 32.3	0.62
	125	227	368		
10. Basal (without zinc in mineral mixture)	125	176	239	111 ± 30.4	0.41
	123	176	232		

[1] Figures shown for average weights represent mean of each pen of 10 chicks. Those for average gains represent the mean of the two replicates, with standard deviations.

[2] Average grams gain per gram of feed consumed. Figures shown represent the mean of the two replicates.

spectively. The addition of 5 p.p.m. of zinc to diet A increased average weight gains from 188 to 218 gm. However, this amount of zinc only partially prevented the growth inhibition brought about by the excessive dietary calcium, whereas 25 p.p.m. zinc completely overcame the inhibition. The addition of zinc to diet A increased the efficiency of feed utilization, while excess calcium depressed feed efficiency. This depressing effect of excess calcium was also only partially overcome

TABLE 5

Effect of addition of zinc or calcium or both to diet A on tibiotarsae [1] of chicks
(Experiment 3)

DIET NO. AND DESCRIPTION	BONE ASH	BONE LENGTH	RATIO LENGTH/ WIDTH	INCIDENCE [2] OF LEG MALFORMATION
	%	*mm*		
1. Basal (diet A)	52.6	55	11.0	8/19
2. + 0.5% Ca	51.9	52		8/20
3. + 1.0% Ca	52.5	52		13/20
4. + 5 p.p.m. Zn	50.8	57		2/20
5. + 0.5% Ca, 5 p.p.m. Zn	51.8	56		7/20
6. + 1.0% Ca, 5 p.p.m. Zn	51.4	53		10/20
7. + 25 p.p.m. Zn	51.2	59	12.1	0/20
8. + 0.5% Ca, 25 p.p.m. Zn	51.4	60		2/20
9. + 1.0% Ca, 25 p.p.m. Zn	51.6	59		1/20
10. Basal (without Zn in mineral mixture)	50.2	50		7/20

[1] Figures given represent mean of both replicates.

[2] Figures given represent number of chicks with malformation/total number of surviving chicks fed each diet.

by 5 p.p.m. of zinc, but was completely overcome by 25 p.p.m. of zinc. The chicks fed diet 10 (diet A with no zinc in the mineral mixture) gained only 111 gm during the experiment, with a low average efficiency of feed utilization of 0.41 gm gain per gram of feed intake.

Data on bone length, bone ash and incidence of a bone malformation found in experiment 3 are summarized in table 5. The bone malformation was characterized by an enlargement and elongation of the tibiotarsal-tarsometatarsal (hock)

joint, similar to that observed by Morrison et al. ('56) in studies on unidentified growth factors. Eight of 19 surviving chicks which received diet A exhibited the condition. The addition of 1.0% calcium to the diet increased the incidence of the syndrome to 13 of 20 surviving chicks. On the other hand, only two of 20 surviving chicks which received an additional 5 p.p.m. of zinc and none of those which received an additional 25 p.p.m. of zinc exhibited the condition. Seven of 20 surviving chicks fed diet 10 (diet A with no zinc in the mineral mixture) exhibited the enlarged hock syndrome. The tibiotarsae of chicks which received diet A were found to be significantly shorter than those of the chicks fed diet A plus 25 p.p.m. of zinc, when the bone lengths were adjusted for differences in body weight by covariance ($p < 0.01$). The results confirm those of O'Dell and Savage ('57) who also found that zinc-deficient chicks had shorter long bones than those which received adequate amounts of zinc. The average ratio of length to width in the tibiotarsae of chicks fed diet A was less than that in the animals which received an additional 25 p.p.m. of zinc (diet 7).

The results of bone ash determinations (table 5) show that the amounts of dietary zinc or calcium had no apparent effect on the percentage ash in the tibiotarsae of the chicks. A similar lack of effect of zinc deficiency on percentage bone ash was observed previously by Day and Skidmore ('47) in the mouse.

The results of experiment 4, conducted to study the effects of the addition of zinc to a diet which contained casein and gelatin (diet B) are summarized in table 6. In contrast to the results obtained with the soybean protein diet (diet A), added zinc had no significant effect on the growth of chicks fed the casein-gelatin diet, even though each diet contained 30 p.p.m. of zinc. Further, addition of 1.0% of calcium to diet B did not depress growth, as was observed with diet A. Addition of both zinc and calcium to diet B resulted in an increase in growth, but this was not significantly greater than that obtained with either zinc or calcium alone. Omission of

TABLE 6

Lack of effect of added zinc on the growth [1] *of chicks fed a diet containing casein and gelatin*
(Experiment 4)

DIET NO. AND DESCRIPTION	AVERAGE WEIGHT AT			AVERAGE GAIN 12–26 DAYS	GAIN OVER BASAL	FEED EFFICIENCY [2] 12–26 DAYS
	12 Days (Initial)	19 Days	26 Days			
	gm	*gm*	*gm*	*gm*	%	
11. Basal (diet B)	119	216	335	219 ± 28.4	—	0.67
	121	215	342			
12. + 25 p.p.m. Zn	118	222	356	237 ± 30.7	8	0.71
	118	223	354			
13. + 1.0% Ca	119	222	350	224 ± 30.0	2	0.65
	120	216	338			
14. + 25 p.p.m. Zn, 1.0% Ca	119	229	372	250 ± 34.1	14	0.70
	119	224	367			
15. Basal (without zinc in mineral mixture)	119	193	295	163 + 42.6	— 26	0.61
	120	195	272			

[1] Figures shown for average weights represent mean of each pen of 12 chicks. Those for average gains represent the mean of the two replicates, with standard deviations.

[2] Average grams gain per gram of feed consumed. Figures shown represent the mean of the two replicates.

zinc from the mineral mixture used in diet B (diet 15) resulted in significantly less growth, 163 gm, as compared with that found on diet B, 219 gm ($p < 0.01$ by variance analysis). This decrease in growth, however, was much less than that observed when zinc was omitted from diet A (experiment 3, table 4).

The average efficiencies of feed utilization observed in experiment 4 (table 6) paralleled the growth results. Omission of zinc from the mineral mixture (diet 15) lowered the feed efficiency slightly, whereas addition of zinc or calcium or both had little effect.

The results of preliminary experiments designed to study the metabolic role(s) of zinc in the chick indicate that the addition of zinc to diet A has no apparent effect on fasting blood sugar levels or on serum pyruvic acid, lactic acid and alkaline phosphatase levels, but results in increased intestinal alkaline phosphatase levels.

The possible relationship of the results obtained in the present experiments to unidentified chick growth factors is of interest. In one experiment it was observed that the addition of 2.5% of dried fish solubles to diet A (protein level kept constant) elicited a 21 gm (10%) increase in weight gains. However, in the presence of 100 p.p.m. of added zinc, no response was obtained from the sample of fish solubles used. Furthermore, fish solubles counteracted the growth depression brought about by addition of 0.5% of calcium to diet A.

The relative ease with which an apparent zinc deficiency was produced in the studies reported herein was somewhat unexpected, in view of the stringent measures usually necessary to produce zinc deficiency in the rat. Because the results obtained by the addition of zinc to the casein-gelatin diet differed so markedly from those obtained with the soybean protein diet, it seems possible that the soybean protein used may have impaired the absorption or utilization of zinc, or in some way have increased the dietary requirement for zinc. Further, the possibility of imbalance in the soybean protein diet must not be overlooked.

SUMMARY

During a 14-day experimental period (from 12 to 26 days of age) the growth of chicks fed a semipurified diet containing soybean protein was significantly increased by addition of zinc to the diet. However, added zinc had no effect on the growth of chicks which received a similar diet containing casein and gelatin as the protein sources. Both diets contained approximately 30 p.p.m. of zinc, 4.8 p.p.m. of which was supplied in the mineral mixture. Chicks which received the soybean protein diet with zinc omitted from the mineral mixture exhibited markedly retarded growth, lowered efficiency of feed utilization and shortened and thickened tibiotarsae. Zinc deficiency had no apparent effect on the percentage of ash in the tibiotarsae or on liver or carcass composition. Omission of zinc from the mineral mixture in the casein-gelatin diet also depressed growth, although the growth depression was not as great as that observed with the soybean protein diet.

The addition of excess calcium to the soybean protein diet depressed weight gain and feed efficiency. Additional zinc, however, counteracted these effects, suggesting that excess calcium may increase the dietary requirement for zinc.

The possible relationship of the above findings to unidentified chick growth factors is discussed.

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CYTOPATHOLOGIC CHANGES IN LIVER CORD CELLS OF ARGININE-DEFICIENT CHICKS[1]

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The arginine requirements of chicks fed purified diets vary with the dietary ingredients used (Krautmann et al., '56). On a purified diet containing 22% of casein, Snyder ('55) and Snyder et al. ('56) found the total requirement to be 1.73%. When the same basal diet was supplemented with 0.3% of L-arginine HCl, making the total arginine 1.06%, the chicks exhibited clinical signs of stunted growth, ruffled feathers, goose-stepping and ataxia. The pathologic basis of the syndrome was not determined. Supplementation of this diet with 0.9% of L-arginine HCl alleviated both the growth deficiency and the ataxia whereas an increase of the magnesium in the basal diet from 0.025 to 0.04% exerted no such influence.

In studying the magnesium requirement of chicks on a 22.2% water-washed casein diet, supplemented with 0.3% of L-arginine monohydrochloride, and containing approximately 0.025% of magnesium, Bird ('48, '49) found the deficiency syndrome to be characterized clinically by poor growth and feathering, fine palpable tremor, forced movements, squatting and ataxia. Pathologic alterations were described in the cerebellar Purkinje cells which exhibited swelling, tigrolysis, nuclear alterations and decreased staining affinity of their dendrites. The feeding of a commerical diet presumably sufficient in magnesium alleviated the ataxia and the occurrence of such pathologic alterations.

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The clinical similarity of the syndromes caused by arginine and magnesium deficiencies on essentially similar purified diets suggested a pathologic comparison which forms the subject of this communication.

MATERIALS AND METHODS

Male chicks from a mating of New Hampshire males and Columbian females were fed the experimental diets in the laboratories of the University of Illinois for three weeks and killed by exsanguination; portions of brain, adrenal, gonad, heart, liver, kidney and spleen were fixed immediately and shipped to the University of Connecticut. In the first experiment tissues were collected from 6 chicks per lot and fixed in 10% formol-saline, in the second experiment from 5 chicks per lot and separately fixed in 2% acetated 10% formalin, Bouin's and Carnoy's. The tissues were processed by the paraffin method and routinely stained with Harris' hematoxylineosin. Special staining techniques applied are discussed in connection with the histochemical observations.

Lot 1 received the basal diet no. 2 (Snyder et al., '56) which was similar to that of Bird ('49) in arginine and magnesium levels; lot 2 was supplemented with 0.15% of $MgSO_4 \cdot 7H_2O$; lot 3 with 0.9% of L-arginine·HCl; and lot 4 with both magnesium and arginine in the same amounts as lots 2 and 3. The basal diet was calculated to contain 1.06% of arginine and 0.025% of magnesium.

HISTOPATHOLOGIC OBSERVATIONS

None of the visceral organs exhibited consistent gross or microscopic changes which could be associated with the experimental treatments, except the liver.

Liver

The initial observation revealed arginine-deficient chicks to present liver cord cells with enlarged nucleoli. This finding was confirmed on chicks fed an unspecified arginine-

deficient diet by Dr. R. White-Stevens, Lederle Laboratories, Pearl River, N. Y. and kindly put at our disposal. In arginine-sufficient chicks the corresponding nuclei failed to present such changes, were similar to those in routine material, and were considered normal. The various fixatives used did not influence these results although Carnoy's fixative increased the chromatic definition of the nucleus.

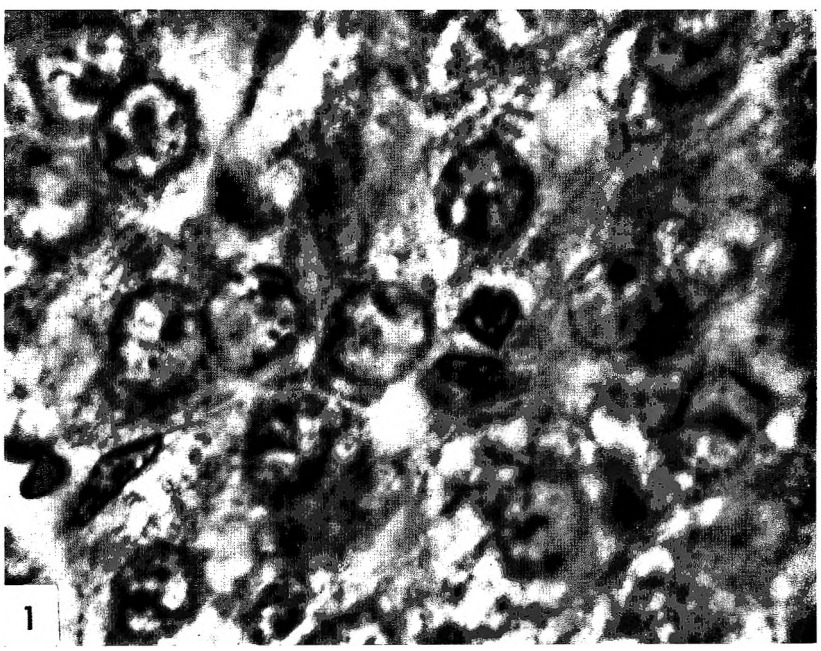

Fig. 1 Photomicrograph of liver section from chick A59492 WB 7368, lot 3, fed the basal diet plus arginine. The liver cord cells are normal. Hematoxylin-eosin. × 2200.

Qualitative changes. The histologic picture of the liver cord cells (fig. 1) from the arginine-supplemented lot 3 corresponded in all respects to the well-known appearance of normal liver cord cells. The cytoplasm within the roundish or hexagonal cells was finely granulated with routine stains. It was known that coarse basophilic particles, consisting essenti-

ally of ribonucleic acid, can be demonstrated by special techniques (Opie, '46; Opie and Lavin, '46). The spherical nucleus was of the typical vesicular type; the nuclear membrane was sharply defined by conspicuous lace-like deposits of chromatin on the inside. The nucleoplasm was delicately granulated and usually contained one small eccentric polygonal nucleolus and several irregularly dispersed chromocenters or aggregated

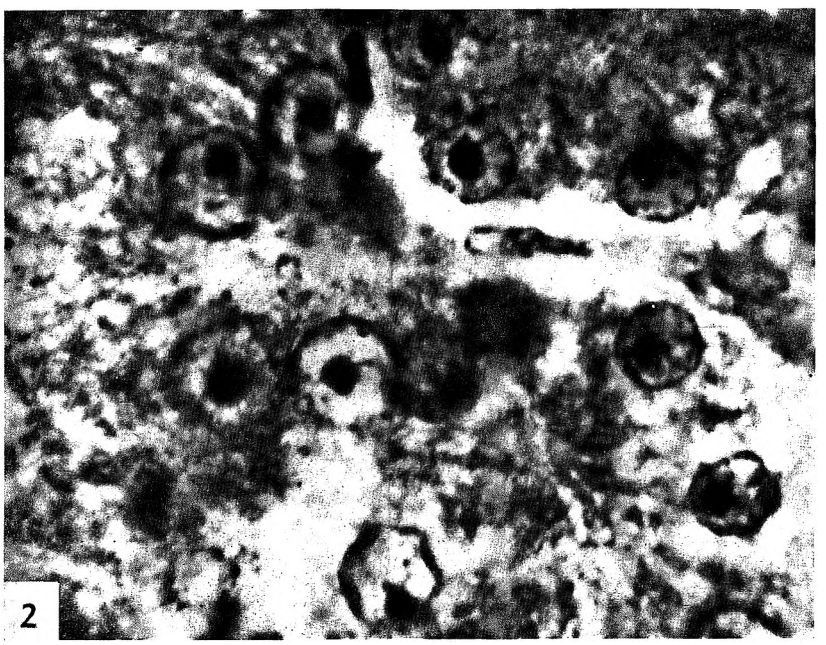

Fig. 2 Photomicrograph of liver section from chick A59490 WB 7342, lot 1, fed the basal diet. The liver cord cells show hypertrophied nucleoli and occasionally hydrodystrophic changes of nucleoplasm. Hematoxylin-eosin. × 2200.

chromomers, in the terminology of Albuquerque and Serra ('51).

The corresponding cells of the arginine-magnesium-supplemented lot 4 presented essentially the same features except that some of the cells exhibited a hydrated effect which is described below for the magnesium-supplemented lot 2.

The liver cord cells of the arginine-deficient lot 1 presented striking abnormalities (fig. 2). Whereas the cytoplasm re-

tained the finely granulated, occasionally vacuolated structure with a somewhat reduced staining intensity, the nucleus appeared enlarged both absolutely and relatively, the latter owing to a thinning of the chromatin deposits on the nuclear membrane. The nucleoplasm lacked the delicate granulation and was almost clear, except for isolated remnants of chromocenters. The nucleolus was enlarged, dense, roundish and nearly centered.

The corresponding cells of the magnesium-supplemented lot 2 exhibited the principal change of nucleolar hypertrophy but, in addition, both the cytoplasm and the nucleoplasm presented a loose reticular structure of decreased stainability, a change which was interpreted as hydrodystrophy. The hydrating effect of magnesium supplementation superimposed on that of arginine deficiency, was not always consistent and could not have been evaluated by itself.

Quantitative changes. On diligent search, normal and abnormal liver cord cells could be found in all lots but in varying proportions. In an attempt to put the diet-induced differences in the appearance of liver cord cells on a quantitative basis 100 nuclei and corresponding nucleoli were measured in 11 slides, a total of 1100 nuclei, per lot. A Bausch and Lomb filar micrometer and a 43 × Zeiss objective were used. The number of nuclei or nucleoli falling into interval groups of 0.2 μ representing one small division on the filar micrometer drum, was ascertained. For convenience the nuclear components were considered circular and the largest diameter at sharp focus to be representative of the size. The distributions of the size of the nuclear components in per cent and the arithmetic means for the 4 lots are shown in figure 3.

It will be seen that the quantitative data bear out the qualitative observations. The nucleoli of the arginine-deficient lot 1 had a mean diameter of 1.82 μ, almost twice that of 0.99 μ for the arginine-supplemented lot 3. The distribution of size

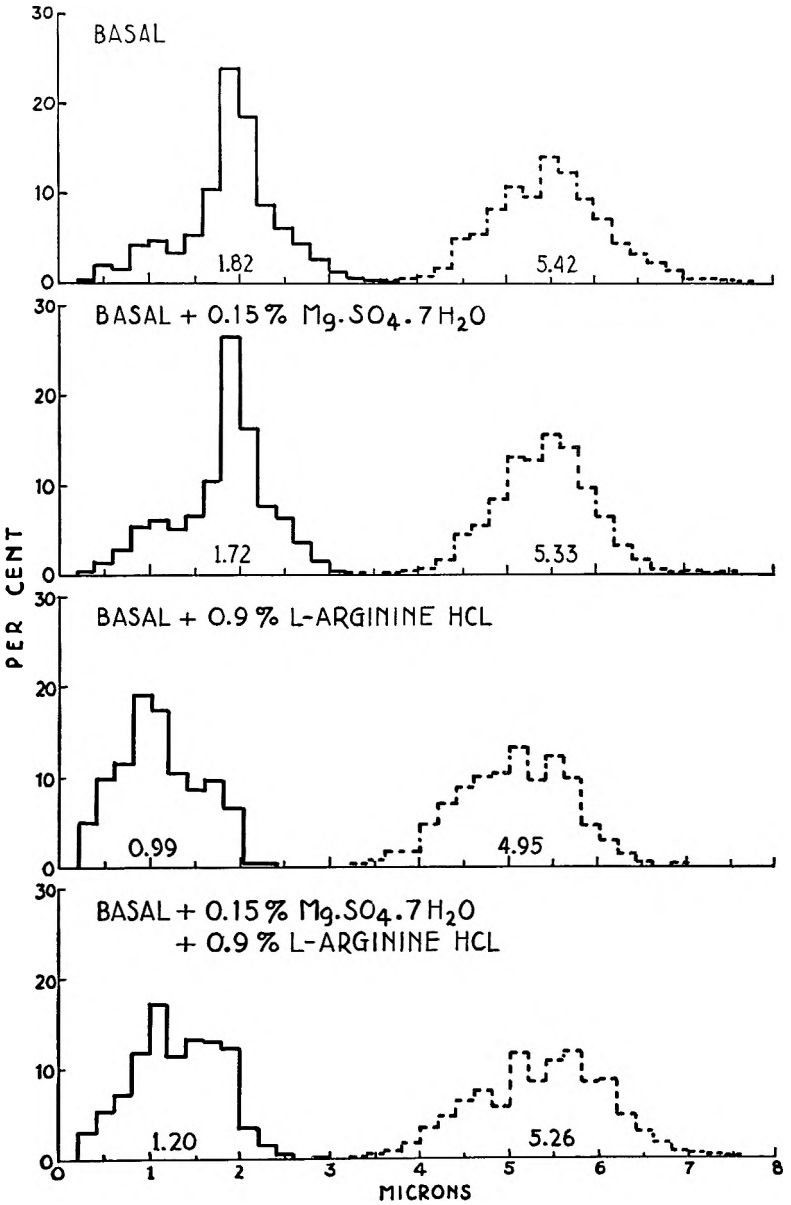

Fig. 3 Size of nucleoli and nuclei of liver cord cells, based on filarmicrometer measurements of 1100 cells; ——— nucleoli, — — — nuclei; insert figure gives average size in microns.

according to the original data for the range 1.6 to 2.1 μ was for lot 1, 53.17; for lot 3, 16.71%; above this range up to 3.7 μ, 24.78 and 0.54%, respectively. Corresponding differences occurred in the magnesium-supplemented lots 2 and 4 but somewhat less striking, presumably owing to the hydrating effect of the element.

The nuclei of the arginine-deficient lot 1 had a mean diameter of 5.42 μ in comparison with 4.95 μ of the arginine-supplemented lot 3. The difference was slight and of borderline significance. Magnesium supplementation of the arginine-deficient lot 2 slightly decreased the average size of the nucleus, contrary to expectations, but showed an increase or hydrating effect for the arginine-supplemented lot 4. Again the differences were slight and probably not significant.

In general, the qualitative and quantitative differences between arginine-deficient and supplemented lots were more striking in the nucleoli than in the nuclei of the liver cord cells; magnesium supplementation seemed to have a superimposed hydrating effect on the cells, which minimized but did not erase, the morphologic effect of the arginine deficiency.

Nucleolar hypertrophy in other conditions. Nucleolar hypertrophy of the liver cord cells is considered a nonspecific lesion. Rather ('51) observed it in routine human autopsy material and in rats fed thioacetamide (a product used for the control of orange decay). Stowell ('48) found this change in regenerating liver cord cells of rats subjected to partial hepatectomy.

The lesion is apparently not a precursor of necrosis, according to Gupta's work ('56) but may represent evidence of polyploidy, i.e., an abnormal increase of chromosomes (Desau and Jackson, '55). In the authors' experience the alteration is uncommon in routine avian necropsy material and in experimental A, D and E hypovitaminoses of chickens.

Histochemical aspects. The nucleolus, according to Albuquerque and Serra ('51), consists of a basic protein, a non-basic protein, nucleic acid of the ribonucleic acid (RNA) type, and a phospholipid which as yet has not been demonstrated

histochemically. The nucleolus is believed to be the site of intense synthetic metabolic activity, and there may be several nucleolar zones in the nucleoplasm which act as nucleolar organizers. The volume of the nucleolus is ordinarily 1 to 10% of that of the nucleus. The dispersed chromocenters contain primarily nucleic acid of the deoxyribonucleic acid (DNA) type.

The question arose whether the large intranuclear body in arginine deficiency was a hypertrophied nucleolus or an aggregate of chromocenters. The tests were applied to representative sections of the arginine-deficient lot 1 and the arginine-supplemented lot 3.

As stated, routine Harris' hematoxylin-eosin permitted ready recognition of the cytologic lesion in the liver cord cells. Toluidine blue, used according to Montagna et al. ('51) and known to stain both RNA and DNA, gave excellent definition; when 10% perchloric acid was used before staining for the extraction of RNA, the large intranuclear body disappeared. Feulgen's test for DNA applied according to Lillie ('51) failed to stain the large body. In Kurnick's ('55) pyronin Y and Korson's ('51) trichrome stain there was excellent definition of the large body. With Korson's ('51) differential stain for RNA and DNA, the cytoplasm appeared orange, the chromocenters blue- green, and the large body purple, the latter color reaction suggesting its nucleolar nature and RNA composition. His enzymatic pretreatment of sections with ribonuclease resulted in disappearance of the large body, whereas deoxyribonuclease left the structure virtually intact.

In general, the histochemical tests used indicated that the large intranuclear body consisted primarily of RNA and represented a hypertrophied nucleolus. Whether the volume increase of the nucleolus was due to its own metabolic activity or to central condensation of RNA from nucleolar organizers dispersed in the nucleoplasm, could only be a matter of speculation. The latter possibility was suggested by the chromatin-depleted appearance of the nucleoplasm. Arginine seemed to

be an important factor for the morphologic integrity of the nucleolus.

Brain

Histologic sections of the brain failed to disclose significant and consistent changes which could be associated with the experimental treatments. In view of Bird's ('48, '49) observations in magnesium-deficient chicks, particular attention was directed to the cerebellar Purkinje cells.

Representative material was subjected to examination with special neuropathologic stains such as Nissl, gallocyanin, and Bodian's protargol technique. Previous experience had indicated that the Purkinje cells in normal avian material are subject to wide morphologic variations, which present difficulties for diagnostic interpretation (Jungherr, '53).

Many of the cerebellar sections revealed Purkinje cells in various stages of alteration as described by Bird. In preliminary observations these changes were not correlated with treatment. To test these points quantitatively an attempt was made to classify the appearance of Purkinje cells in routine hematoyxlin sections into "normal," "pyknotic," "otherwise abnormal," and "missing" cells. The latter classification was introduced in order to account for interruptions of regular spacing. By means of this classification a differential count was made of 200 Purkinje cells from 6 representative chicks, a total of 1200 per lot. Only straight-line rows of Purkinje cells, parallel to the central white matter of the folia, were counted because the cell arrangement near the peripheral curves was always somewhat disturbed. The data obtained for the 4 classes of Purkinje cells were so nearly alike for the 4 lots as to preclude interpretation. In the overall, our material failed to disclose any specific lesions in the cerebellar Purkinje cells of either arginine- or magnesium-deficient chicks, correctable by these substances, alone or in combination.

DISCUSSION

In comparison with the large number of known nutritional deficiencies there are relatively few such entities with a

definite pathologic basis, not overshadowed by secondary factors of inanition or infection (Follis, '48). The best example, of course, is squamous metaplasia which is almost pathognomonic for A-hypovitaminosis. It seems that there exist "target organs," or those most likely to exhibit the earliest specific lesion. These organs may vary with the species. For example A-hypovitaminosis is first recognizable in the chicken at the mucocutaneous junction of the nasal septum (Jungherr, '43) but in the ox at the mucocutaneous junction of the parotid gland duct (Jungherr et al., '50). With respect to deficiencies of chemical elements or essential amino acids the knowledge of their pathology is fragmentary. The pathognomonic character of cerebellar Purkinje cell lesions in magnesium deficiency of chicks (Bird, '48, '49) requires confirmation.

The observation of cytopathologic changes in the liver cord cells of chicks on an arginine-deficient diet correctable by arginine supplementation, is of interest from various aspects: (1) It represents one of the few known pathologic bases of a single amino acid deficiency. (2) The characteristic change of nucleolar hypertrophy has been seen previously in other animals, but only as a result of toxic, not of nutritional, factors. This hypertrophy appears to be in excess from the increase in nucleolar mass which goes proportionately with the synthetic activity of the cell (Schultz, '52). (3) The lesion is reminiscent of basophilic intranuclear inclusion bodies in certain virus diseases e.g. canine infectious hepatitis and opens up speculation as to the contribution of viral and nutritional factors to the formation of such inclusion bodies. (4) Since the basophilic body in the affected liver cord cells seems to be composed primarily of RNA and to represent a hypertrophied nucleolus it would appear that — in the chick — arginine is involved in the maintenance of the normal morphologic integrity of the hepatic nucleolus.

It would be of interest to know whether the same amino acid deficiency induces similar lesions in other species or other amino acid deficiencies similar lesions in the same species.

An important question is also whether borderline arginine deficiency in chickens induces lesions of diagnostic value.

SUMMARY

The liver cord cells of chicks fed a purified 22% casein, low-magnesium, low-arginine diet showed cytopathologic changes characterized by a large intranuclear central basophilic body accompanied by hydrodystrophic changes of the nucleoplasm and the cytoplasm. Histochemical tests suggested that the large body consisted primarily of ribonucleic acid and represented a hypertrophied nucleolus. The hepatic lesion was correctable by arginine. There were no consistent lesions in the cerebellar Purkinje cells referrable to either arginine deficiency or the lowest level of magnesium fed. Supplementation with magnesium accentuated the hydrodystrophic alteration of the liver cord cells, but failed to correct the nucleolar hypertrophy. The latter lesion has been observed previously in rats fed toxic doses of thioacetamide or acetamide, but not in nutritional deficiencies.

The possible implications of these findings, with respect to the formation of basophilic intranuclear inclusion bodies and the role of arginine in the morphologic integrity of the nucleolus, are discussed.

ACKNOWLEDGMENTS

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STUDIES OF THE EFFECTS OF DIETARY SODIUM FLUORIDE ON DAIRY COWS

III. SKELETAL AND SOFT TISSUE FLUORINE DEPOSITION AND FLUORINE TOXICOSIS [1]

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The completion of a long-term study of the effects of a soluble fluoride (NaF) on the development of a chronic fluorine toxicity in dairy cattle (Suttie et al., '57, '58) has made available material for an extensive study of fluorine accumulation and its effect on osseous and soft tissues of the animal body.

The body has two main defensive mechanisms against increased dietary levels of fluorine. A limited amount of ingested fluorine can be rapidly excreted in the urine, and a large portion is deposited in the skeleton as fluoroapatite (Phillips, Greenwood, Hobbs and Huffman, '55) and is thus effectively removed from the fluids bathing the soft tissues. Because of these means of protection, the fluorine concentration of the blood and soft tissues remains very low even under conditions of relatively toxic fluorine intakes (Chang, Phillips, Hart and Bohstedt, '34; Hobbs, Moorman, Griffith, West, Merriman, Hansard and Chamberlain, '54).

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Since fluorine storage in the skeleton is dependent on the total fluorine ingested, the fluorine concentration of the bones provides a definitive criterion in the diagnosis of a chronic fluorine toxicosis.

This report presents data on the accumulation of fluorine in the soft and osseous tissues of cattle when known levels of a soluble fluoride were fed for an extended period.

EXPERIMENTAL

Two-year-old Holstein heifers which had been bred were assigned to 6 lots on the basis of the amount of NaF added to the ration. Lot 1 was fed the basal dairy ration which contained 3 to 5 p.p.m. of fluorine; lots II, III, IV, V and VI were fed the basal ration plus 20, 30, 40, 50 and 60 p.p.m. of fluorine, respectively. To the ration of lot VI was also added 200 gm of $CaCO_3$ per day.

The basis of this allotment and the general husbandry practices employed have been described (Suttie et al., '57). The fluorine was administered as a solution poured over the daily grain ration. The cattle were slaughtered during their 6th lactation after 5½ years of controlled exposure to dietary fluorine.

At slaughter any gross pathology of internal organs was noted, and a representative sample of the heart, liver, kidney, pancreas, adrenal and thyroid was obtained for fluorine analysis. Portions of the same tissues were fixed in Bouin's solution for histological examination. A blood sample was also obtained and frozen for analysis. The metacarpal, metatarsal, mandible, maxilla, and frontal bones, as well as the 11th and 12th ribs were obtained for fluorine analysis and study.

After cleaning, the bones were inspected for any gross evidence of abnormality and the metacarpus and metatarsus were photographed.

ANALYTICAL PROCEDURE

Samples of soft tissue were cut into narrow strips and dried at 95 to 105°C for 24 hours. Heart, liver and kidney samples were then ground to pass a 40-mesh screen in a micro Wiley

mill. The pancreas, thyroid and adrenal glands were extracted 24 hours with ether in a Goldfisch continuous extractor before grinding. After grinding, all samples were stored in glass-stoppered vials in a desiccator.

For the fluorine determination, a 5-gm sample of the ground tissue (less than 5 gm for some thyroid and adrenal samples) was weighed into a platinum dish and mixed with one gram of low-fluorine CaO (6.85 p.p.m. F). The sample was then mixed with 50 ml of redistilled water and boiled to dryness to ensure alkalinity of the entire sample and to give a partial digestion. The samples were charred under heat lamps, transferred to a muffle furnace, and ashed at 1100°F for 10 hours. For the blood fluorine determination, one gram of CaO and 25 ml of redistilled water were added to 25 ml of whole blood, and treated as described above.

The fluorine distillation of the ash from soft tissues was carried out in the all-glass system devised by Richter,[3] and 40 ml of the 100-ml distillate titrated by conventional methods (Alcoa Research Laboratory, '47; Willard and Winter, '33).

A representative sample of the entire length of the metacarpus and metatarsus was obtained by making a median lateral bisection and collecting the particles made by the saw. The frontal bone was also sampled by making a series of transverse saw cuts. A trephine was used to obtain a post mortem rib sample identical to that obtained by previous biopsy (Suttie et al., '57). To avoid variation the trabeculae (the more metabolically active bone) were scrapped off and only the dense rib bone was used.

The bone samples were extracted with ether for 24 hours in a Goldfisch continuous extractor, dried, and analyzed for fluorine by the Alcoa Research Laboratory modification ('47) of the Willard and Winter method ('33).

RESULTS

Data on the fluorine concentration of the bones are summarized in table 1. It is evident that the 3 to 5 p.p.m. of fluorine

[3] Unpublished data, E. F. Richter, WARF Laboratory, Madison, Wisconsin.

in the diet of the control animals resulted in considerable deposition of fluorine in the skeleton. These data indicate that the skeletal structures of normal dairy cows, as represented by lot I, contained less than 1000 p.p.m. of fluorine. More specifically, the metacarpal-metatarsal bones contained an average of 584 p.p.m., the rib and frontal bones 661 p.p.m. of fluorine. Apparently the cancellous bones of the body, as

TABLE 1

The effects of added dietary increments of F⁻(NaF) upon bone fluorine concentrations in dairy cows

LOT	F ADDED	COW	METACARPAL [1]	METATARSAL [1]	FRONTAL [1]	12TH RIB [1]
	p.p.m.		*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
I	0	2	593	482	647	694
		3	878	647	701	635
		4	463	436	592	703
		Av.	645	522	645	677
II	20	5	2720	2610	3200	3290
		6	2770	2990	3340	4770
		7	2170	2610	3110	4030
		8	2660	3030	3430	3910
		Av.	2580	2810	3270	4000
III	30	10	4180	4090	4540	4900
		11	3780	3310	4920	5970
		12	4120	4200	4800	5280
		Av.	4030	3870	4750	5380
IV	40	13	5520	5280	6180	7030
		14	5840	5380	6010	7070
		15	4510	4710	—	4100
		16	3740	3180	4730	4860
		Av.	4900	4640	5640	5770
V	50	17	7610	7800	8260	9000
		19	5470	4920	6800	8100
		20	4050	4360	6700	6870
		Av.	5710	5690	7250	7990
VI	50 + $CaCO_3$	22	4950	5320	7420	8640
		23	5800	5820	7050	9250
		24	5030	4490	6700	7830
		Av.	5260	5210	7060	8570

[1] Dry fat-free weight.

represented by those of the skull and ribs, concentrate F more readily than the compact bones of the legs. This differential spread increased as higher levels of fluorine were added to the diet.

It is apparent that the bone fluorine concentrations increased progressively with each added increment of 10 p.p.m. of dietary fluorine. Increments of added fluorine at levels of 20 or 30 p.p.m. fluorine (NaF) resulted in leg bone depositions of 4.5 and 6.7 times those of the controls (lot 1). These levels were without apparent harm since the cows suffered no other untoward effects and did not show any clinical symptoms of fluorine toxicosis during the test period of 5½ years. Supplementation with NaF equivalent to 40 p.p.m. of fluorine caused fluorine deposition of more than 8 times the amount present in similar bones of the control cows. Since a few mild fleeting manifestations of developing fluorine toxicosis were observed in certain animals thus exposed (Suttie et al., '57), this level may be considered the marginal zone of fluorine toxicosis under 5½-year test conditions.

It was found that the addition of 50 p.p.m. of fluorine to the ration resulted in fluorine toxicosis in two out of the three animals in the group. At the time of slaughter, cow 17 which had shown debilitating toxicosis during the third year and recovered was on the verge of another collapse. In this cow the deposition of skeletal fluorine exceeded 7500 p.p.m. for all bones analyzed. For the entire group (lot V, 50 p.p.m. F) it was found that the skeletal fluorine storage was about 10 times that of the controls. Although there were indications that the addition of excess calcium to the ration decreased the absorption of fluorine and somewhat mitigated its toxic effect (Suttie et al., '57), this did not affect the concentration of fluorine in the bones when 200 gm of $CaCO_3$ per day were added to the ration of the cows in lot VI which received 50 p.p.m. of fluorine.

Inspection of the data in the light of the physiological responses previously discussed (Suttie et al., '57) revealed that bone fluorine concentrations in toxicosis were in excess of

5500 p.p.m. for the leg bones and above 7000 p.p.m. for the more metabolically active cancellous bone structures. Concentrations ranging downward from 5500 to 4500 p.p.m. of fluorine apparently was the marginal zone, while levels between the normal range of 1000 and 4000 p.p.m. were innocuous and without direct physiological significance. It is widely accepted that fluorine is a cumulative poison. Therefore both the level at which the fluorine was fed and the duration of the exposure are pertinent to the development of fluorine toxicosis. Thus the time interval required to build up skeletal flourine concentrations from a normal value of less than 1000 p.p.m. to a marginal level of 5000 p.p.m. represents the "latent period" of Shortt and co-workers ('37) and others (Phillips and Suttie, '57).

In contrast to the skeletal deposition, the fluorine concentration of the "soft tissues" in cows fed 50 p.p.m. of fluorine was very low. The data in table 2 indicate that the normal fluorine concentration of the soft tissues as represented by the control group was 2 to 3 p.p.m., which was approximately 10 times that present in whole blood. It appears that this very low concentration of fluorine was raised 2 to 3-fold when the diet contained more than 30 p.p.m. fluorine. Cow 17 (lot V, 50 p.p.m. F) which was the only animal suffering from fluorine toxicosis at the time of slaughter had no higher fluorine concentrations in the soft tissues than other cows in lot V. The kidney values reported were representative samples of the entire kidney and therefore contained considerable residual urine which would in part explain the higher fluorine concentration in this organ. Present methods of fluorine analysis do not completely rule out distillation of iodine with the fluorosilisic acid,[4] and the subsequent interference of iodine in the visual titration. Because of this, accuracy of thyroid fluorine

[4] It was observed that the distillate from some thyroid samples was not clear, but had a slight brown tint. The contaminant was identified as iodine by the violet color in a chloroform extraction of the distillate, and in some cases by a positive reaction with starch paper. Preliminary results indicate that there is no loss of fluorine if the distillate is extracted with chloroform prior to titration.

TABLE 2

The effect of added dietary increments of F⁻(NaF) upon soft tissue fluorine concentrations in dairy cows

LOT	F ADDED	COW	HEART [1]	LIVER [1]	KIDNEY [1]	PANCREAS [2]	THYROID [2]	ADRENAL [2]	BLOOD [3]
	p.p.m.		*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
I	0	2	1.8	3.1	3.1	1.7	0.6	2.0	0.49
		3	3.3	1.9	2.9	4.0	2.7	11.9	0.32
		4	1.7	1.9	4.4	2.0	2.9	2.2	0.22
		Av.	2.3	2.3	3.5	2.8	2.1	5.3	0.34
II	20	5	2.7	2.4	7.3	1.4	2.9	3.8	0.34
		6	4.4	2.1	6.0	2.6	7.0	—	0.59
		7	2.5	2.3	8.3	3.2	6.6	—	0.84
		8	4.0	3.9	12.8	1.7	11.2	3.3	0.39
		Av.	3.4	2.7	8.6	2.2	6.9	3.5	0.54
III	30	10	2.7	2.5	12.5	2.0	2.4	3.3	0.45
		11	4.7	4.6	9.1	5.0	4.1	—	0.89
		12	3.0	5.1	10.5	3.5	4.1	3.3	0.30
		Av.	3.5	4.1	10.7	3.5	3.5	3.3	0.55
IV	40	13	3.3	5.6	19.7	3.0	4.9	2.5	0.94
		14	5.5	3.3	20.4	5.1	5.0	3.5	0.69
		15	4.5	3.4	7.8	—	—	—	—
		16	2.9	3.0	16.0	3.4	7.6	8.8	0.31
		Av.	4.0	3.8	16.0	3.8	5.8	4.7	0.66
V	50	17	5.0	2.3	13.7	4.5	5.2	8.7	0.38
		19	3.2	2.1	15.4	4.1	12.2	—	0.78
		20	6.3	4.8	28.9	4.0	4.4	4.1	0.84
		Av.	4.6	3.6	19.3	4.2	7.3	6.4	0.67
VI	50 + $CaCO_3$	22	3.8	3.5	11.1	3.6	4.2	—	1.10
		23	4.6	2.7	7.7	3.5	4.9	4.2	0.68
		24	5.6	2.8	8.4	3.0	9.0	4.1	0.88
		Av.	4.6	3.0	9.0	3.4	6.0	4.1	0.89

[1] Dry weight. [2] Dry, fat-free weight. [3] Whole blood.

values may be open to question. This study serves to illustrate that the fluorine concentration of the soft tissues, because of the narrow margin between normal and fluorosed tissue, is a very poor index of the toxic effects due to fluorine ingestion.

Pathologic conditions of the bones have been observed in fluorine toxicity (Shupe et al., '55; Dale and Crampton, '55). The data on the occurrence and severity of exostosis and ankylosis of the metacarpus and metatarsus, arbitrarily evaluated by numbers from 1 to 5, are summarized in table 3. A rating of 1 was given to a slight visible exostotic area or areas

TABLE 3

The effects of added dietary increments of fluorine (NaF) upon the calcification of bones and joints of dairy cows

LOT	F ADDED	COW	DEGREE OF EXOSTOSIS [1]		DEGREE OF CARTILAGE CALCIFICATION [1]	
			Metacarpus	Metatarsus	Metacarpus	Metatarsus
	p.p.m.					
I	0	2	0	0	0	0
		3	0	0	0	0
		4	0	0	0	0
II	20	5	0	0	1	1
		6	0	0	0	0
		7	0	0	0	0
		8	0	0	0	1
III	30	10	1	1	1	3
		11	0	0	1	1
		12	1	2	2	3
IV	40	13	1	2	2	2
		14	1	2	1	2
		15	1	1	0	1
		16	2	2	0	1
V	50	17	4	5	2	5
		19	3	3	2	4
		20	4	4	2	3
VI	50 + $CaCO_3$	22	4	4	1	4
		23	4	4	2	4
		24	3	4	1	2

[1] Based on a rating of 0 for normal bone and 5 for the most severely effected.

discernible in a thoroughly cleaned bone while a number 5 represented the most severe condition which caused a marked overgrowth and general thickening of periosteal bone. The metatarsal bones were more severely affected than the front leg bones. The data indicate the appearance of the condition beginning in the cows fed 30 p.p.m., and becoming progressively more severe with added increments of dietary fluorine. The severity and degree of exostoses below number 4 was not palpable or visible in situ. Further, an exostosis-like condition was encountered from bruising and trauma especially of the hocks and knees and in a few cases without direct relation to added dietary fluorine.

Periarticular tissues, including the fascia and to some extent cartilage, were observed to have undergone calcification progressively with the severity of exostosis exhibited by the metacarpus and metatarsus bones (fig. 1 and table 3). The physiological cause of this phenomenon is unknown. The affinity of these tissues for calcium suggests that fluorine either directly inhibits the normal calcification control system in these structures or sensitizes them to calcium deposition.

Associated with the appearance of well-defined leg-bone exostosis there was a thickening of the mandible which seemed to exert a twisting stress that resulted in a flatter, less-arched incisor bed. The skull bones were also affected. The hard palate arch was flattened slightly and the nasal bone arched to give the face a "Roman Nose" appearance.

The data herein presented concerning skeletal changes induced by added dietary fluorine support the accepted concept that exostosis and ankylosis of the long bones of the legs are symptoms associated with fluorine toxicosis in cattle. However, exostosis as a diagnostic tool in the differentiation of variable degrees of toxicity in the living animal was of little value except in the more advanced cases.

From the completeness with which warm detergent solutions defatted them, it appeared that the exostotic bones were more porous and easily penetrated than normal bones. The

increased porosity and softness was in general associated with bones containing more than 4000 p.p.m. of fluorine.

The viscera of all cattle were examined at the time of slaughter and no gross pathology due to fluorine ingestion was observed.

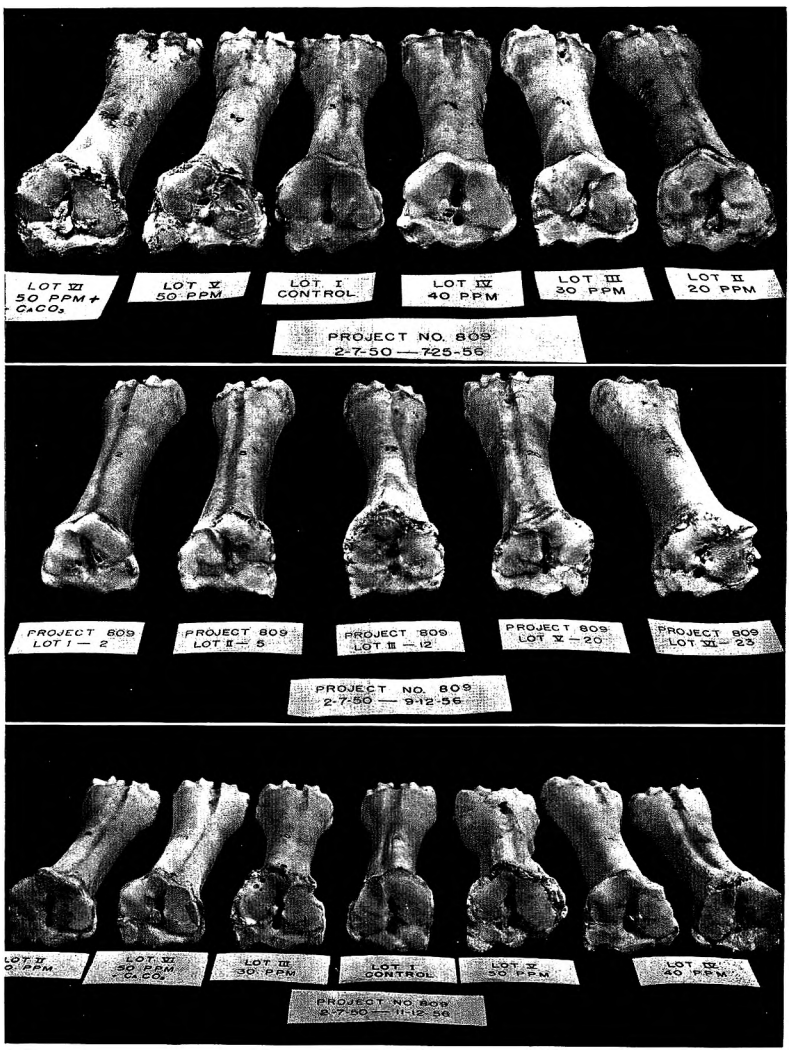

Fig. 1 The effects on the metatarsal bones of cows fed graded levels of fluorine (NaF). The type and extent of exostosis and calcification of periarticular tissue are evident.

SUMMARY

The effects of added increments of dietary fluorine in the form of sodium fluoride upon skeletal deposition and subsequent changes have been studied in dairy cows fed for 5½ years a basal ration which contained 3 to 5 p.p.m. fluorine. A study of the data obtained appears to establish the following facts.

1. Dairy cows fed the basal ration stored less than 1000 p.p.m. of fluorine in the bones of the skeleton.

2. There was a progressive increase in the fluorine content of bone with each added increment of dietary fluorine. The increase was 4.5 and 10 times that of the controls when 20 p.p.m. and 50 p.p.m. of fluorine were added, respectively.

3. The bone fluorine content varied with the type of bone; cancellous bone was uniformly higher in fluorine than the compact leg bones from the same animal.

4. Flourine toxicosis in the dairy cow was associated with a fluorine content of compact bone and of cancellous bone in excess of 5500 and 7000 p.p.m. respectively. Concentrations ranging downward from 5500 to 4500 p.p.m. of fluorine seemed to provide a marginal zone while levels below 4500 p.p.m. were innocuous. The time interval required to increase bone (leg) fluorine concentrations from 1000 to over 5000 p.p.m. represents the "latent period" in these animals.

5. The fluorine content of the soft tissues from the control cows was found to be 2 to 3 p.p.m. and these values were increased two- to three-fold by adding 50 p.p.m. of dietary fluorine to the basal ration. The narrow margin in concentrations between normal and fluorosed tissues makes the use of soft tissue analyses as a criterion of fluorine toxicosis an unreliable one.

6. Mild to extensive exostosis developed in the metacarpal and metatarsal bones which was more pronounced in the latter. Slight exostosis on a tissue-free bone was observed in cows of lot III when the bones of the legs containing 4000 or more p.p.m. of fluorine.

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PHOSPHATE AVAILABILITY STUDIES WITH THE ASH OF UNIDENTIFIED GROWTH FACTOR SUPPLEMENTS

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A number of reports have appeared in recent literature on the involvement of both known and unidentified nutrients in bone calcification in animals. Morrison et al. ('56) reported that an unidentified mineral(s) was involved in bone formation. They found that the inclusion of the mixed ash of 5 unidentified growth factor supplements in the diet significantly increased the percentage of bone ash and the breaking strength of the tibiotarsal bones of chicks that received the diet for 4 weeks. Although their basal diet contained the amounts of both calcium and phosphorus recommended by the National Research Council ('54), no evidence was presented by these workers to show that the bone changes observed in their studies might not have been produced by the additional phosphorus and calcium present in the ash of the 5 unidentified growth factor supplements. Scott et al. ('56), and Scott ('57) also presented data on the effect of the ash of 4 unidentified growth factor supplements on calcification in turkey poults. These workers concluded from their results that the ash of the unidentified growth factor supplements was not only involved in calcification but that it was intricately involved in the utilization of phosphorus from certain phosphorus supplements by poults. O'Dell and Savage ('57) recently described the production of zinc deficiency in the chick. They reported that zinc-deficient chicks had shortened and thickened long

bones with a reduced ash content of the dry fat-free tibia, indicating that zinc is involved in the calcification process. The work presented in this report was carried out to determine what effect the ash of unidentified growth factors might have on the availability of various phosphates to the turkey poult and the chick.

EXPERIMENTAL

Turkeys. Day-old Broad Breasted Bronze male turkeys were distributed into 24 groups of 10 poults each and placed in electrically heated battery brooders with wire mesh floors. Feed and water were supplied ad libitum. Weight gain and feed consumption were recorded weekly. At the end of a three-week experimental period the poults were sacrificed and the left tibiae removed for bone ash determination (Association of Official Agricultural Chemists, Methods of Analysis, '55).

The composition of the basal diet used in this experiment, expressed as grams per 100 gm of diet, was as follows: cornstarch, 21.60; low fiber soybean oil meal, 67.0; DL-methionine, 0.65; glycine, 0.50; cellulose,[1] 3.0; hydrogenated vegetable fat,[2] 7.0; plus the following amounts of vitamins and minerals expressed as milligrams per 100 gm of diet: butylated hydroxytoluene (BHT), 50.0; niacin, 8.81; calcium pantothenate, 2.20; riboflavin, 0.99; pyridoxine·HCl, 0.99; thiamine·HCl, 0.99; folic acid, 0.22; biotin, 0.022; choline chloride, 247.5; *d*-alpha tocopheryl acetate concentrate N.F. (250 mg/gm), 26.43; menadione, 1.98; vitamin A concentrate (5000 USP units/gm), 264.32; vitamin D_3 concentrate (15000 ICU/gm), 29.29; KCl, 700.0; NaCl, 900.0; $MnSO_4 \cdot H_2O$, 50.0 $MgSO_4$, 244.0; $FeSO_4 \cdot 5H_2O$, 20.0; $ZnCl_2$, 1.0; $CoCl_2, \cdot 6H_2O$, 0.4. The phosphorus content of this basal diet was 0.55%. The various phosphate supplements were added to the diet at the expense of starch and the Ca:P ratio was maintained at 2:1 in all diets by the addition of $CaCO_3$.

[1] Solka Floc. The Brown Company, Berlin, New Hampshire.

[2] Primex, Procter and Gamble Company, Cincinnati, Ohio.

Chicks. Day-old Single Comb White Leghorn cockerels were handled in the same manner as the turkey poults. The number of chicks per lot varied from experiment to experiment and is indicated in the respective tables. The basal diet used in the chick experiments reported herein contained the following ingredients expressed as grams per 100 gm: corn starch, 66.74; blood fibrin, 20.0; gelatin, 4.0; cellulose,[1] 3.0; liver fraction "L," 1.0; hydrogenated vegetable fat,[2] 3.0; vitamin A concentrate (5000 USP units/gm), 0.20; NaCl, 0.75; KCl, 0.60; $MgSO_4$, 0.255; *d*-alpha tocopheryl acetate concentrate N.F. (250 mg/gm), 0.07; choline chloride, 0.20, plus the following expressed as milligrams per 100 gm; inositol, 110.0; para-aminobenzoic acid, 11.0; calcium pantothenate, 2.20; niacin, 2.64; thiamine·HCl, 1.32; riboflavin, 1.32; pyridoxine·HCl, 0.66; folic acid, 0.44; menadione, 0.22; biotin, 0.044; vitamin B_{12} 0.022; vitamin D_3 concentrate (15, 000 ICU/gm), 13.332; $MnSO_4 \cdot H_2O$, 26.4; $FeSO_4 \cdot 7H_2O$, 11.0; $CuSO_4 \cdot 5H_2O$, 1.1; $CoCl_2 \cdot 6H_2O$, 1.1; $ZnCl_2$, 1.1; KI, 1.1; $Na_2MoO_4 \cdot 2H_2O$, 0.11. This diet contained approximately 0.05% phosphorus. The supplements were added at the expense of cornstarch and the Ca : P ratio of each diet was maintained at 2 : 1 by the addition of $CaCo_3$.

RESULTS AND DISCUSSION

Experiment 1, turkeys. This experiment was carried out to determine if the ash of certain unidentified growth factor supplements (UGF ash)[3] would affect the availability of inorganic phosphates to turkeys. Dicalcium phosphate (U. S. P.),[4] a phosphate readily available for turkeys, was fed at 4 levels to obtain a standard curve. Curacao Island rock phosphate (hereafter referred to as Curacao rock phosphate) and c.p. dicalcium phosphate,[5] relatively unavailable phosphate

[1] See footnote, page 306.

[2] See footnote, page 306.

[3] Equal parts of dried distillers' solubles, dried whey, fish solubles and penicillin mycelium meal were ashed individually; the ash was then combined to form the unidentified growth factor ash (UGF ash).

[4] U.S.P. dicalcium phosphate is the hydrated salt, $CaHPO_4 \cdot 2H_2O$.

[5] C.P. dicalcium phosphate is the anhydrous salt $CaHPO_4$.

materials for turkey poults, were fed at two phosphate levels to determine the availability of these materials. Phosphorus from UGF ash and potassium acid phosphate were fed at two levels. The availability of the phosphorus from USP dicalcium phosphate, c.p. dicalcium phosphate and Curacao rock phosphate was also determined in the presence of either the UGF ash or potassium phosphate. The series with potassium phosphate was run as a control against the UGF ash, each being fed at comparable levels of phosphorus. The growth results and the percentage of tibia ash are shown in table 1.

On the whole, good growth was obtained in this experiment, except in a few lots which received relatively unavailable phosphate. The lower level of c.p. dicalcium phosphate, Curacao rock phosphate or the ash of the UGF all gave poor growth when used as the sole source of supplementary phosphorus. Also, when c.p. dicalcium phosphate and the Curacao rock phosphate were combined with the UGF ash at the lower level of phosphorus supplementation, poor growth was obtained.

The data on percentage of bone ash have been graphically summarized and are shown in figure 1. The addition of graded levels of phosphorus from U.S.P. dicalcium phosphate produced a gradient increase in calcification. Except for potassium phosphate, which was just as available as the U.S.P. dicalcium phosphate, the other sources of phosphorus were not as available as the U.S.P. dicalcium phosphate to turkey poults. If a value of 100 is assigned to the U. S. P. dicalcium phosphate, then the c.p. dicalcium phosphate would have a biological value of 58; the Curacao rock phosphate, 50 and the phosphorus from the UGF ash, a biological value of 64.

The addition of the UGF ash to the U.S.P. dicalcium phosphate lowered the bone ash values as compared to those obtained from the U.S.P. dicalcium phosphate alone. The addition of the UGF ash to c.p. dicalcium phosphate, or the Curacao rock phosphate, gave higher bone ash values than were obtained from either the c.p. dicalcium phosphate or the

Curacao rock phosphate alone. However, this increase in bone ash was proportional to that expected when a poorly available source of phosphorus is added to one of higher availability. The addition of potassium phosphate to U.S.P. dicalcium phosphate had no effect on the bone ash values obtained, while the addition of potassium phosphate to c.p. dicalcium

TABLE 1

Effect of ash of four unidentified growth factor supplements (UGF ash) upon utilization of phosphorus by the turkey poult

TREATMENT	AVERAGE WEIGHT 3 WKS.	TIBIA ASH
	gm	*%*
0.195% P, U.S.P. dical [1]	408	38.6
0.26% P, U.S.P. dical	426	43.7
0.39% P, U.S.P. dical	440	46.5
0.455% P, U.S.P. dical	402	46.7
0.26% P, c. p. dical [2]	378	35.5
0.39% P, c. p. dical	394	42.0
0.26% P, Curacao [3]	373	34.2
0.39% P, Curacao	407	38.6
0.26% P, UGF ash [4]	347	37.9
0.39% P, UGF ash	389	43.3
0.26% P, c. p. KH_2PO_4	441	43.4
0.39% P, c. p. KH_2PO_4	439	47.2
0.13% P, U.S.P. dical + 0.13% P, UGF ash	407	40.7
0.26% P, U.S.P. dical + 0.13% P, UGF ash	399	45.5
0.13% P, c. p. dical + 0.13% P, UGF ash	381	36.5
0.26% P, c. p. dical + 0.13% P, UGF ash	418	40.7
0.13% P, Curacao + 0.13% P, UGF ash	360	35.6
0.26% P, Curacao + 0.13% P, UGF ash	390	39.3
0.13% P, U.S.P. dical + 0.13% P, c. p. KH_2PO_4	424	43.7
0.26% P, U.S.P. dical + 0.13% P, c. p. KH_2PO_4	449	47.0
0.13% P, c. p. dical + 0.13% P, c. p. KH_2PO_4	418	40.4
0.26% P, c. p. dical + 0.13% P, c. p. KH_2PO_4	426	±4.1
0.13% P, Curacao + 0.13% P, c. p. KH_2PO_4	419	±0.4
0.26% P, Curacao + 0.13% P, c. p. KH_2PO_4	415	±2.6

Source:

[1] U.S.P. dicalcium phosphate.

[2] C. P. dicalcium phosphate.

[3] Curacao Island rock phosphate.

[4] Unidentified Growth Factor ash (see footnote 3, pg. 307).

phosphate or to the Curacao rock phosphate caused an increase in bone ash values. Again, these values are in line with those expected in view of the availability of the phosphorus in potassium phosphate.

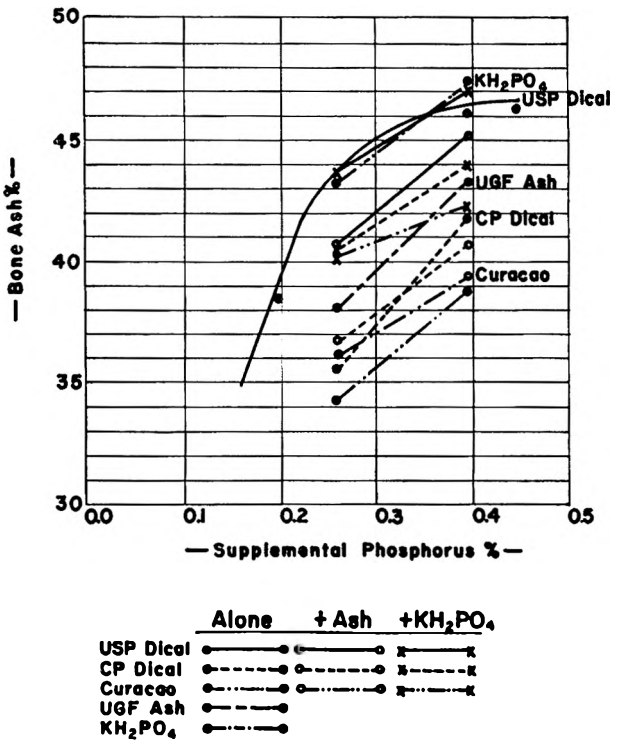

Fig. 1 Effect of unidentified growth factor ash and KH_2PO_4 on the utilization of phosphorus from Curacao Island rock phosphate and c.p. dicalcium phosphate by turkey poults.

The biological values of the various materials used in this study, assigning a value of 100 to U.S.P. dicalcium phosphate, can be used to predict the biological values expected when mixtures of the materials are fed. A comparison of such predicted values with values actually obtained by measuring the percentage of bone ash of turkey poults fed the various mixtures is presented in table 2. The values obtained from the mixtures were almost identical with those predicted from

the values obtained with single sources of the pure materials. These results show that the presence of the UGF ash or potassium phosphate does not influence the availability of phosphorus from U.S.P. dicalcium phosphate, Curacao rock phosphate, or c.p. dicalcium phosphate to the turkey poult.

TABLE 2

Predicted vs. obtained biological values of the combinations of phosphatic materials fed to turkey poults in experiment 1

TREATMENT	BIOLOGICAL VALUE	
	Predicted	Obtained
0.13% P, U.S.P. dical [1] + 0.13% P, UGF ash [2]	82	82
0.26% P, U.S.P. dical + 0.13% P, UGF ash	81	88
0.13% P, c. p. dical [3] + 0.13% P, UGF ash	72	61
0.26% P, c. p. dical + 0.13% P, UGF ash	55	60
0.13% P, Curacao [4] + 0.13% P, UGF ash	68	57
0.26% P, Curacao + 0.13% P, UGF ash	52	54
0.13% P, U.S.P. dical + 0.13% P, c. p. KH_2PO_4	100	100
0.26% P, U.S.P. dical + 0.13% P, c. p. KH_2PO_4	100	100
0.13% P, c. p. dical + 0.13% P, c. p. KH_2PO_4	81	79
0.26% P, c. p. dical + 0.13% P, c. p. KH_2PO_4	69	72
0.13% P, Curacao + 0.13% P, c. p. KH_2PO_4	81	75
0.26% P, Curacao + 0.13% P, c. p. KH_2PO_4	62	66

[1] U.S.P. dicalcium phosphate.
[2] Unidentified Growth Factor ash.
[3] C. P. dicalcium phosphate.
[4] Curacao Island rock phosphate.

The superior availability of the hydrated dicalcium phosphate as compared with the anhydrous material is probably a reflection of differences in rate of solution and crystal structure. The high degree of availability of monopotassium phosphate has been demonstrated repeatedly. It is completely soluble in water or dilute acid solutions.

Experiment 2, chicks. In this experiment the effect of the UGF ash on the availability of the phosphorus in Curacao rock phosphate to Single Comb White Leghorn cockerels was studied. The experimental design and results are presented

in table 3. The UGF ash, c.p. potassium phosphate, and Curacao rock phosphate were each fed to supply three levels of phosphorus, namely, 0.15, 0.25 and 0.35%. The Curacao rock phosphate was then fed at two levels in the presence of both

TABLE 3

Effect of UGF ash on the availability of the phosphorus from a poorly available source to the chick [1]

TREATMENT	AVERAGE WEIGHT 3 WKS.	TIBIA ASH	MORTALITY
	gm	*%*	*%*
0.15% P, UGF ash [2]	122	25.3	35
0.25% P, UGF ash	194	32.1	5
0.35% P, UGF ash	206	39.6	5
0.15% P, c. p. KH_2PO_4	178	28.9	15
0.25% P, c. p. KH_2PO_4	218	38.8	5
0.35% P, c. p. KH_2PO_4	224	44.3	0
0.15% P, Curacao [3]	—	—	100
0.25% P, Curacao	134	28.3 [4]	55
0.35% P, Curacao	174	31.7	20
0.15% P, UGF ash + 0.10% P, c. p. KH_2PO_4	214	36.7	5
0.15% P, UGF ash + 0.20% P, c. p. KH_2PO_4	223	44.2	0
0.15% P, UGF ash + 0.10% P, Curacao	176	29.9	10
0.15% P, UGF ash + 0.20% P, Curacao	194	35.1	5
0.15% P, c. p. KH_2PO_4 + 0.10% P, Curacao	204	34.5	15
0.15% P, c. p. KH_2PO_4 + 0.20% P, Curacao	207	38.7	0

[1] Twenty White Leghorn cockerels per treatment.

[2] Unidentified Growth Factor ash.

[3] Curacao Island rock phosphate.

[4] This value not used in the calculation of the biological value due to the high mortality in this group.

the UGF ash and c.p. potassium phosphate. Potassium phosphate was also fed at two levels in the presence of the UGF ash. The experiment was designed to measure the increase in bone ash that would result from the addition of 0.10% of phosphorus from Curacao rock phosphate, both in the absence and presence of the UGF ash. However, high mortality occurred in the Curacao rock phosphate lots at the two lower levels

of supplementary phosphorus. One hundred per cent mortality occurred among the chicks that received 0.15% phosphorus from this source and 55% of those that received 0.25% phosphorus died. In view of this high mortality at the 0.25% phosphorus level, the 28. 3% bone ash value that was obtained from the 9 surviving chicks in this group has very little meaning, since those chicks which survive usually have higher bone ash values than the group as a whole. Therefore, only the bone ash data from the group receiving 0.35% of phosphorus from the Curacao rock phosphate was used to calculate the biological value.

TABLE 4

Predicted vs. obtained biological values of the combinations of phosphatic materials fed to chicks [1] in experiment 2

TREATMENT	BIOLOGICAL VALUE	
	Predicted	Obtained
0.15% P, UGF ash + 0.10% P, c. p. KH_2PO_4	88	83
0.15% P, UGF ash + 0.20% P, c. p. KH_2PO_4	98	88
0.15% P, UGF ash + 0.10% P, Curacao	64	63
0.15% P, UGF ash + 0.20% P, Curacao	58	59
0.15% P, c. p. KH_2PO_4 + 0.10% P, Curacao	79	80
0.15% P, c. p. KH_2PO_4 + 0.20% P, Curacao	71	73

[1] Single-comb White Leghorn cockerels.

In this experiment, the biological values were based on potassium phosphate which was assigned a value of 100 with the result that Curacao rock phosphate had a value of 50 and the unidentified growth factor ash, a value of 72. As in the previous experiment, the biological values of the individual materials were used to calculate the expected values when various mixtures of the phosphate sources were fed. A comparison of the predicted biological values versus the biological values actually obtained for the various mixtures of phosphates is shown in table 4.

It is evident that the presence of either the unidentified growth factor ash or potassium phosphate does not have an

effect on the availability of the phosphorus in Curacao rock phosphate.

Experiment 3, chicks. This experiment was carried out to determine specifically if the blood fibrin diet is deficient in zinc for the young chick. The basal diet used in this study was the same as that in the previous experiments except that $ZnCl_2$ was omitted. The experimental outline and results are presented in table 5 . These data reflect no improvement in growth or calcification from the addition of 5 or 100 p.p.m. of zinc to the blood fibrin diet when it contained either a sub-optimal

TABLE 5

Effect of zinc on chick growth and bone ash at two phosphorus levels using a blood fibrin-gelatin diet

ZINC ADDED [1]	PHOSPHORUS ADDED [2]	AVERAGE WEIGHT 3 WKS.	TIBIA ASH
p.p.m.	%	*gm*	%
0	0.30	210	40.6
5	0.30	206	40.9
100	0.30	204	40.1
0	0.60	215	44.1
5	0.60	215	44.7
100	0.60	228	44.4

[1] Added in the form of $ZnCl_2$.

[2] Added in the form of U.S.P. dicalcium phosphate.

(0.30%) or an adequate (0.60%) amount of supplemental phosphorus. The addition of 5 p.p.m. of zinc to the diet made it similar to the diets used in the previous experiment. This lack of response from zinc was expected since the blood fibrin used in the diet contained 988 p.p.m. of zinc by analysis. Although UGF ash (1,620 p.p.m. zinc by analyses) would be expected to improve growth and bone formation on a zinc-deficient diet, the blood fibrin-gelatin type diet is apparently quite adequate in this element. The results of these experiments also show that zinc does not significantly improve the availability of a poorly available phosphate.

SUMMARY

Experiments were conducted to determine whether the ash of unidentified growth factor supplements would affect the availability of inorganic phosphates to turkey poults or chicks. The results of the turkey experiment showed that:

1. Supplementation with the unidentified growth factor ash does not increase the availability of phosphorus from Curacao Island rock phosphate or c.p. dicalcium phosphate to the turkey poult.

2. The relative biological values of various materials for turkeys (assigning U.S.P. dicalcium phosphate a value of 100) were: c.p. dicalcium phosphate 58, Curacao Island rock phosphate 50, unidentified growth factor ash 64, and c.p. monopotassium phosphate 100.

3. The percentages of bone ash in the tibia of turkeys fed combinations of these supplements approximated those predicted from the relative availabilities of the individual phosphates.

The results of the chick experiments showed that:

1. The presence of the unidentified growth factor ash did not influence the availability of phosphorus in a relatively unavailable phosphate, such as Curacao Island rock phosphate.

2. No improvement in growth or percentage bone ash was obtained from the addition of zinc to the blood fibrin-gelatin diet. This diet contains a sufficient amount of zinc to meet the chick's requirement.

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EFFECTS OF SEMISTARVATION ON THE DISTRIBUTION OF ERYTHROCYTES AND PLASMA IN ORGANS AND TISSUES OF THE RAT [1]

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The published reports relative to changes in blood volume during starvation indicate that plasma volume increases during prolonged semistarvation and decreases in acute starvation, (Keys et al., '50). Although the circulating red cell volume has not been measured directly following starvation, it is generally agreed that red cells and hemoglobin are resistant to change. However, anemia eventually ensues and, as pointed out by Keys et al. ('50) this anemia may be masked by severe weight loss and dehydration. These alterations in total body blood volume have not been related to individual organ and tissue blood volumes. The numerous studies reported of changes in individual organs subsequent to starvation have concerned primarily such selective changes as those of size, weight and morphology (Morgulis, '23).

Since no reports have been made of organ and tissue blood volumes subsequent to starvation the present study was undertaken utilizing methods developed in this laboratory and reported previously for the normal rat (Everett et al., '56).

METHODS

Inanition was produced by the methods of Rivero-Fontan et al. ('52) since these investigators provided an analysis of the morphological changes in rat organs and tissues following

[1] This investigation was supported in part by a research grant H-1530 from the National Heart Institute, U. S. Public Health Service.

starvation. Briefly, two isocaloric diets [2] were used, — A with adequate protein and B which had 4 times the protein content of A. During the starvation period of two weeks, rats were given 25% of their previously established normal daily food intake. Twenty-four Sprague-Dawley male rats which had received a commercial food [3] ad libitum and greens once weekly were placed on each experimental diet. These animals had an average weight of 240 gm. This weight was selected so that after starvation the weights would be in the range of those used in the normal series whose organ blood volumes were reported earlier (Everett el al., '56) and serve as controls for this study. Hematocrit determination of venous blood obtained by cardiac puncture were made for 8 rats on each starvation diet. The remaining 16 rats in each group were divided equally for red cell and plasma volume determinations. The methods used including injection of tagged cells, or albumin, freezing in liquid nitrogen, organ dissection, sample preparation and counting, and calculations have been reported in detail (Everett et al., '56). A brief description of these methods follows. Rat erythrocytes tagged with Fe^{59} were used for the red cell measurements and I^{131} serum albumin was used for the plasma determinations. The rats were frozen in liquid nitrogen at three minutes after injection of I^{131} albumin or 10 minutes after administration of Fe^{59} erythrocytes. The rats were dissected in the frozen state to avoid blood loss; the organs and tissues were weighed and assayed for radioactivity in a well-type scintillation counter, and calculations of cellular, plasma and true blood volumes were made by the following formulae:

[2] Diet A contained, by weight, 72% dextrose, 16% casein, 8% corn oil and 4% salt mixture. Diet B contained 24% dextrose, 64% casein, 8% corn oil and 4% salt mixture (U. S. P. XII, no. 2). Vitamin supplements were added to each diet.

[3] Purina Fox Checkers.

Fe^{59} red cell dilution

$$\frac{\text{activity/gm tissue}}{\text{activity/mg heart blood}} = \text{mg blood/gm tissue}$$

red cell volume (RCV)

$$(\mu\text{l red cells/gm tissue}) = \frac{\text{mg blood/gm tissue}}{\text{density of rat blood (1.056)}} \times \text{hematocrit (HCT) of heart blood}$$

I^{131} albumin dilution

$$\frac{\text{activity/gm tissue}}{\text{activity/mg heart blood}} = \text{mg blood/gm tissue}$$

$$\text{plasma volume (PV) } (\mu\text{l plasma/gm tissue}) = \frac{\text{mg blood/gm tissue}}{1.056} \times \text{1-HCT of heart blood}$$

Combining I^{131} and Fe^{59} values

$$\text{true blood volume (TBV) } (\mu\text{l blood/gm tissue}) = \text{red cell volume (RCV)} + \text{plasma volume (PV)}$$

$$\text{tissue hematocrit} = \frac{\text{RCV}}{\text{TBV}}.$$

RESULTS

Table 1, listing changes in the total body weight, shows losses ranging from 25 to 30% with no significant differences between animals on adequate (A) and those on high (B) protein intake. The mean hematocrit values for fresh cardiac blood were 48.5 and 50.9 respectively for rats on diets A and B. The hematocrit of the control series was 41.5, (Everett et al., '56).

TABLE 1

Body weight loss on starvation diets

BLOOD COMPONENT MEASURED	NO. RATS	INITIAL WEIGHT	FINAL WEIGHT	LOSS	CV
		gm	*gm*	*%*	
		Diet A (low protein)			
Red cells	8	249	175	29.9	9.3
Plasma volume	8	242	183	24.4	5.5
Hematocrit	8	234	165	29.5	5.3
		Diet B (high protein)			
Red cells	8	244	179	26.5	7.7
Plasma volume	8	239	176	26.3	1.6
Hematocrit	7	234	165	29.5	6.1

TABLE 2

Tissue blood volumes of rats [1] on starvation diets

	Fe^{59} RED CELL MEASUREMENTS						I^{131} ALBUMIN MEASUREMENTS						COMBINED DATA					
	Controls RCV		Diet A RCV		Diet B RCV		Controls PV		Diet A PV		Diet B PV		Controls TBV	HCT [2]	Diet A TBV	HCT	Diet B TBV	HCT
	µl/gm	*cv* [3]	*µl/gm*	*cv*	*µl/gm*	*cv*	*µl/gm*	*cv*	*µl/gm*	*cv*	*µl/gm*	*cv*	*µl/gm*		*µl/gm*		*µl/gm*	
Total rat	20.4	1.3	25.3	1.7	26.2	2.2	35.2	2.9	34.5	6.1	32.4	2.0	55.6	36.7	59.8	42.3	58.6	44.7
Adrenal	46.8	5.9	54.6	7.4	65.6	8.9	191.0	1.7	117	9.1	147	3.0	238.0	19.7	172	31.7	213	30.8
Bone	5.6	2.5	25.6	6.3	23.7	16.9	34.9	1.1	54.6	10.5	59.5	5.7	40.5	13.8	80.2	31.9	83.2	28.5
Cerebral hemisphere	8.8	4.4	12.0	2.5	12.4	3.4	21.2	3.4	17.7	2.0	16.9	3.9	30.0	29.3	29.7	40.4	29.3	42.3
Thalamus + midbrain	11.1	4.0	13.2	3.3	16.6	8.2	20.7	4.9	17.8	6.2	18.5	4.6	31.8	34.9	31.0	42.6	35.1	47.3
Cerebellum	12.3	2.8	15.5	3.2	15.2	5.0	25.5	3.9	20.8	6.1	19.8	3.4	37.8	32.5	36.3	42.7	35.0	43.4
Pons + medulla	6.6	6.8	7.85	4.3	8.87	9.6	16.0	3.7	12.7	5.7	12.0	6.9	22.6	29.0	20.6	38.1	20.9	42.4
Myocardium	71.7	3.3	125	2.7	131	5.7	191.0	1.7	160	3.4	154	4.0	262.0	27.1	285	43.9	285.0	46.0
Kidney	28.7	7.1	28.1	4.3	28.8	3.9	99.7	5.7	92.7	9.1	98.1	3.2	128.0	22.4	121	23.2	127	22.7
Liver	82.1	3.1	67.1	6.3	63.1	9.4	188.0	2.3	139	3.5	160	4.1	270.0	30.5	206	32.6	223	28.3
Lung	179.0	3.3	289	2.0	308	1.5	340.0	1.7	333	1.4	313	1.2	519.0	34.5	622	46.5	621	49.6
Seminal vesicle	8.7	8.5	7.49	10.6	10.2	15.9	14.3	7.6	17.1	17.0	16.7	14.4	23.0	37.6	24.6	30.4	26.9	37.9
Skeletal muscle	8.4	3.1	9.74	6.4	10.0	4.5	17.4	3.5	15.5	5.3	15.7	4.4	25.8	32.4	25.2	38.6	25.7	38.9
Skin	7.3	4.5	5.65	11.1	5.78	5.5	11.9	3.2	10.3	4.3	9.02	6.5	19.2	37.9	16.0	35.3	14.8	39.1
Small intestine	8.9	6.0	9.46	8.4	10.3	7.7	25.0	6.4	22.6	4.2	20.9	7.4	33.9	26.3	32.1	29.5	31.2	33.0
Spinal cord	7.0	6.8	6.25	5.2	5.88	14.4	18.1	7.0	12.3	7.0	9.95	9.5	25.1	27.9	18.6	33.6	15.8	37.2
Spleen	86.1	3.7	40.9	15.9	34.8	11.8	83.6	3.6	65.4	6.5	65.6	6.6	170.0	50.7	106	38.6	100	34.8
Submaxillary gland	25.3	9.4	25.7	10.2	26.9	14.5	56.0	8.1	37.2	10.4	39.2	11.2	81.3	31.1	62.9	40.9	66.1	40.7
Testis	4.2	3.4	5.79	4.8	5.16	6.1	11.5	3.1	9.46	5.4	9.62	6.7	15.7	26.5	15.2	38.1	14.8	34.9
Thyroid	95.6	15.5	84.5	22.5	83.9	9.5	85.9	4.6	148	7.8	104	16.3	181.0	52.7	232	36.4	188	44.6

[1] Ten or more rats in each control group, 7 or 8 in each experimental group.

[2] Tissue hematocrit.

[3] Coefficient of variation (σm as % of mean).

The mean blood values including plasma volume, red cell volume and true blood volume for the total rat and for individual tissues and organs are shown in table 2.

These values are expressed in microliters per gram of tissue. In addition the tissue hematocrits are given in table 2. For the rat as a whole, there was some increase in red cell volume per unit weight and a small reduction of plasma resulting in a true blood volume (PV + RCV) only slightly higher than normal (table 2).

For the most part the blood volume changes of the individual organs and tissues corresponded to this same pattern, that is, an increase in red cell volume and a decrease in plasma volume. Certain tissues, however, showed changes in red cell or plasma volumes or both which were not in line with the general pattern and show differences that are statistically significant (1% level by the *t* test) from the control values. These include adrenal, bone, cardiac muscle, liver, lung, skin, spinal cord, spleen, sub-maxillary gland and testis (fig. 1). In some of these the changes were apparently related to the protein content of the diet.

The RCV of the myocardium and lung increased on either diet and the PV declined resulting in an increase of the true blood volume (TBV) (fig. 1). Although the RCV of the adrenal increased in animals on either diet, the plasma volume decrease was of such a magnitude that the TBV decreased significantly. This decrease was less in the case of the higher protein diet B.

Three organs, spleen, liver, and skin had reduced red cell and plasma volumes (fig. 1). Of these the spleen showed the greatest loss which was 60% and 52% respectively for red cells on diets B and A. The loss in plasma volume for the spleen was 22% on either diet. In the liver the reduction of plasma exceeded that of red cells on diet A but not on diet B. The red cell and plasma volume losses for the skin were approximately of the same magnitude and were comparable in animals on either diet.

DIET A

(lower protein content)

	↑ RCV ↓		↑ PV ↓		↑ TBV ↓		↑ HCT ↓	
Bone	462		156		198		231	
Myocardium	176			83.8	109		162	
Lung	161			97.9	120		135	
Testis	139			82.3		97.0	143	
Cerebral Hemispheres	136			83.5		99.0	138	
Total Rat	125			92.3	102		122	
Cerebellum	126			81.6		96.	131	
Pons & Medulla	120			79.4		90.4	132	
Thalamus & Midbrain	119			86.0		97.4	122	
Skeletal Muscle	116			89.1		97.8	119	
Adrenal	117			61.2		72.3	161	
Small Intestine	106			90.4		94.6	112	
Submaxillary Gland	102			66.4		77.4	131	
Kidney		97.9		92.9		94.4	104	
Seminal Vesicle		86.6	120		107			80.8
Liver		81.7		73.9		76.3	104	
Skin		77.6		86.6		84.		93.4
Spleen		47.5		78.2		62.5		75.7

DIET B

	↑ RCV ↓		↑ PV ↓		↑ TBV ↓		↑ HCT ↓	
Bone	425		171		205		206	
Myocardium	184			80.6	109		170	
Lung	172			92.1	120		144	
Testis	124			83.7		94.3	132	
Cerebral Hemispheres	141			79.7				
Total Rat	128			92.	105		122	
Cerebellum	124			77.7		92.6	124	
Pons & Medulla	135			75.0		92.5	145	
Thalamus & Midbrain	150			89.4	110		161	
Skeletal Muscle	120			90.2		99.6	120	
Adrenal	140			77.0		89.1	156	
Small Intestine	116			83.6		92.0	126	
Submaxillary Gland	106			70.0		81.3	131	
Kidney	100			98.4		99.2	101	
Seminal Vesicle	118		117		117		101	
Liver		76.8		85.1		82.6		92.8
Skin		79.5		75.8		77.1	103	
Spleen		40.4		78.5		58.8		68.6

Fig. 1 Blood values of organs from animals on diets A and B expressed as percentage of control values. The direction of the arrows indicate increase or decrease from the control values.

The most extreme changes in blood volume occurred in bone (femur and tibio-fibula combined). Here the RCV of animals on either diet averaged more than 4 times that of the controls. The RCV increased 360%, the PV 56%, the TBV 98%, and the hematocrit 131% (fig. 1) on diet A. There was a slightly greater plasma rise (70%) and lower gain of erythrocytes (325%) when more protein was ingested.

It is perhaps significant that the blood values of the kidney were not altered by either starvation diet.

The ratio of the hematocrit of the whole rat to that of blood from the heart was 0.878 on diet A and 0.872 on diet B. In the control series this ratio was 0.884.

DISCUSSION

The increase in erythrocytes on a unit-weight basis is a reflection of the concentrating effect of the body weight loss rather than an acquisition of red cells. These rats at an average starting weight of 247 gm would have a total of 5.04 ml of red cells as calculated from the normal control values (Everett et al., '56). Following the two-week starvation period their average total cell volume measured 4.5 ml. With respect to plasma, the slightly lower volume on a unit weight basis would obviously indicate an appreciable drop in the total supply of plasma. These observations are in accord with the report of Keys et al. ('50) that in man anemia eventually ensues following either acute or semi-starvation and as shown here may be masked by body weight loss. The decrease in plasma volume reported here, although small, follows the pattern described for man by Taylor et al. ('54) during acute starvation.

At the level of caloric intake used here no significant difference in the relation between the TBV of the total rat and the amount of protein ingested was shown; possibly because the need for calories was such that the protein was largely consumed to provide them. That selective differences, dependent on the amount of protein or protein-derived substances did exist however is suggested in the following observations:

The RCV was somewhat higher in seminal vesicles, and thalamus and mid-brain when more protein was ingested.

The adrenal and liver had higher plasma volumes on the diet with more protein.

Conversely the lung and skin contained more plasma when the intake of protein was restricted.

Inasmuch as these data were computed on a unit-weight basis they are not merely a reflection of the loss of weight of the organ or tissue studied. With this in mind it appears that the general reduction in plasma volume tends to be offset by an increase in the volume of erythrocytes in the maintenance of homeostasis. The mechanism by which the different structures effected this change varied for reasons as yet unknown. One may speculate that the loss of red blood cells by the spleen, liver and skin of the semistarved animals may have resulted in the gain of erythrocytes by other tissues. Inasmuch as the reciprocal relationship of erythrocytes and plasma shown by the total rat is not necessarily reflected in the individual organs and tissues, changes in PV may in certain instances be a reflection of alteration of capillary permeability due to starvation.

It is known that in acute semistarvation the loss of fat from most of the common depots is rapid and far exceeds that of other tissues such as bone which is remarkably stable in weight and mineral composition, (Keys et al., '50). In the experiments reported here the loss of fat from the marrow, enveloped by a rigid structure incapable of shrinking about the potential space left by the disappearing fat, might well be responsible for the great influx of blood into the bone cavities. As the fat was mobilized a replacement that was rapidly available was needed. An influx of blood may have supplied this.

Analogous arguments might explain in part the increased TBV in the lungs and heart inasmuch as they are enclosed within a semi-rigid cage which in the case of the lungs would tend to hold their size constant. As mediastinal fat is lost the capacity of the thorax is increased. Initially this relatively greater capacity may be partly filled with blood. Later

emphysema may appear (Keys et al., '50). Undoubtedly stability of the total circulating blood volume is also an important factor here inasmuch as a serious reduction in this volume would have been reflected in the blood content of the heart and lungs regardless of the rigidity of the chest.

Elsewhere in the body the possible mechanisms effecting the circulatory changes are less clearly defined because, with the exception of the contents of the cranium and spinal canal, the tissues are free to shrink around one another as the fat is lost. That other factors do exist, however, is evident from the diverse responses of the liver, certain parts of the brain, the adrenal, the skin, lung, small intestine and submaxillary gland in animals on diets containing different amounts of protein. It is conceivable that the greater content of plasma found in lung, skin and small intestine when less protein was ingested may be related to observed incidence of edema in these particular structures during more prolonged starvation or during the early rehabilitation period after starvation (Keys et al., '50).

SUMMARY

The distribution of erythrocytes tagged with Fe^{59} and plasma labeled with I^{131} was studied in rats under conditions of semi-starvation on diets of equal caloric value containing either adequate amounts of protein or 4 times that amount. It was found that, after an experimental period of two weeks, the total blood volume of the rats per unit of weight changed very little. At this time, however, the amounts of blood in certain organs and tissues differed significantly from those in normal rats. For example, the increased content of erythrocytes in bone, myocardium, lung, testis and some parts of the central nervous system was noteworthy. Elsewhere the predominant change was in the amount of plasma present.

The hematocrit of the rat as a whole increased approximately 20% regardless of the amount of protein in the diet, but in certain organs changes in the content of erythrocytes and plasma varied with the amounts of protein ingested. In

this regard, the kidney, during that type of semistarvation, showed great stability. Its content of both plasma and red cells remained almost constant.

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EVALUATION OF PUMPKIN SEED MEAL AS A SOURCE OF PROTEIN FOR SWINE USING A DEPLETION-REPLETION TECHNIQUE [1]

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A reliable estimation of the value of a protein source for practical swine rations, by a conventional growth experiment, requires a considerable quantity of the respective protein source. As supply was limited, a depletion-repletion technique was used for the evaluation of the protein quality of expeller-extracted pumpkin seed oil meal.[4] Swine and rat experiments were conducted simultaneously using the same technique to compare the relative feeding value of pumpkin seed oil meal and expeller-extracted soybean oil meal.[5]

EXPERIMENTAL

Rats. Thirty-two growing rats averaging 93 gm body weight were fed individually a protein-free ration (table 1) ad libitum for a period of 8 days, during which an average weight loss of approximately 20% occurred. The animals (16 males and 16 females) were then randomly allotted by weight within sex to the 4 ration treatments. Soybean oil meal (SBOM) or

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[4] Expeller-processed, 40% crude protein, 22.3% crude fiber, 9.9% crude fat, 1.7% ash and 20.9% nitrogen-free extract; supplied by Central Iowa Bean Mill, Gladbrook, Iowa.

[5] Expeller-processed, 42.6% crude protein.

pumpkin seed oil meal (PSOM) was fed once daily in amounts equivalent to 1.0 or 1.5 gm of crude protein with free access to the protein-free ration which had been used for depletion. Consumption measurements of the protein-free diet were not taken. The protein allowance was readily consumed, except in a few cases on the high level of PSOM; but these were insignificant and no corrections were made.

Swine. Forty-eight crossbred pigs averaging 35 lbs. body weight and 63 days of age were individually fed a protein-free

TABLE 1

Ration composition for protein depletion of rats and for depletion and repletion of swine

INGREDIENT	DEPLETION RATIONS		12% PROTEIN SWINE REPLETION RATIONS [2]	
	Rat [1]	Swine [2]	Semi-purified basal	Corn basal
	%	%	%	%
Cornstarch	30.0	65.0	52.5	—
Dextrose	59.0	10.0	5.0	—
Sucrose	—	10.5	5.0	—
Corn	—	—	—	85.9
Pumpkin seed oil meal [3]	—	—	30.0	9.0
Lard	4.0	2.5	1.0	—
Beet pulp	—	4.0	—	—
Wood flock	2.0	2.0	1.0	—
Dicalcium phosphate	2.5	3.4	2.5	1.8
Calcium carbonate	—	—	0.4	0.7
Salt (Iodized)	0.4	0.5	0.5	0.5
Trace mineral premix [4]	0.1	0.1	0.1	0.1
Vitamin-antibiotic premix	2.0	2.0	2.0	2.0

[1] Calculated vitamins per pound of diet: vitamin A, 4500 I.U.; vitamin D_2, 900 I.U.; thiamine, 3.6 mg; riboflavin, 1.8 mg; niacin, 9.1 mg; calcium pantothenate, 3.6 mg; pyridoxine, 1.4 mg; alpha-tocopherol acetate, 45 mg; folic acid, 0.5 mg; menadione, 1.4 mg; para-amino benzoic acid, 0.5 mg; vitamin B_{12}, 4.5 μg.

[2] Calculated vitamins and antibiotic per pound of diet: vitamin A, 2000 I.U.; vitamin D_2, 500 I.U.; thiamine, 3 mg; riboflavin, 3 mg; niacin, 20 mg; calcium pantothenate, 6 mg; pyridoxine, 1.2 mg; alpha-tocopherol acetate, 10 mg; choline, 400 mg; folic acid, 9 μg; menadione, 3 mg; para-amino benzoic acid, 8 mg; vitamin B_{12}, 10 μg; chlortetracycline, 10 mg.

[3] Soybean oil meal replaced pumpkinseed oil meal in nitrogen equivalent amounts in the rations for half the animals.

[4] Contributed the following in milligrams per pound of complete ration: Fe, 32.0; Cu, 2.2; Co, 0.8; Zn, 37.1; Mn, 25.8; K, 3.4.

ration (table 1) ad libitum for a period of 12 days. Thirty-two animals with similar weight changes were selected and divided into 4 groups according to body weight. The animals were randomly allotted within each replication to the ration treatments. The 8 ration treatments consisted of a $2 \times 2 \times 2$ factorial arrangement of two types of diets (semi-purified and conventional) fed at two levels of protein (12% and 16%) in which the protein was from two sources (soybean oil meal and pumpkin seed oil meal). The ration ingredients were adjusted to approximate the same calculated level of minerals, trace minerals, vitamins and antibiotics. The composition of the protein-free and 12% protein pumpkin seed oil meal rations are presented in table 1. The animals were confined to individual metabolism cages with feed and water provided ad libitum for the 10-day repletion period.

Analysis of data. The repletion gain data for the rats and pigs and the feed required per pound of gain data for pigs were subjected to an analysis of variance as described by Snedecor ('56, chapters 10 and 11). Statements concerning statistical significance refer to a probability level of 5% or less.

RESULTS AND DISCUSSION

Rats. A summary of the repletion gains made by rats is presented in figure 1. On the average, rats fed PSOM as the source of protein gained 44% less than those fed SBOM. The performance on the higher level of protein (1.5 gm/day) was superior to that on the lower level (1.0 gm/day) within each source of protein. However, increasing the daily allowance of PSOM protein improved gains only slightly, whereas increasing the allowance of SBOM protein increased total gain markedly. These differences between sources of protein, levels of protein, and the source $\times$ level interaction were statistically significant.

Swine. The results obtained with swine (table 2 and fig. 2) correspond well with those obtained with rats, here again showing a definite inferiority of PSOM as compared to SBOM. The average repletion gain on the PSOM rations was 62% less

than that on the SBOM rations. Increasing the level of protein in the SBOM rations from 12 to 16% improved gains markedly, whereas increasing the level of protein in the PSOM rations resulted in a slight depression in gains. This interaction between source of protein and level of protein, as well as the above mentioned difference between sources of protein, was statistically significant.

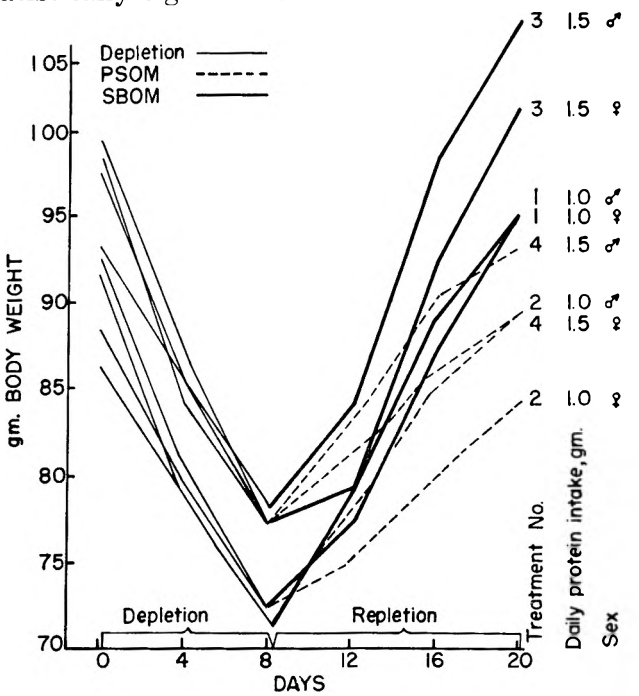

Fig. 1 Body weight changes in rats during depletion and during repletion on two levels of soybean oil meal and pumpkin seed oil meal protein.

The marked difference between the sources of protein was also reflected in the feed required per pound of repletion gain. The pigs fed the SBOM rations required significantly less feed to produce a pound of gain than did the pigs fed the PSOM rations. Increasing the level of protein in the SBOM (both types) rations and in the conventional type PSOM rations improved feed conversion; however, increasing the level of protein in the semi-purified PSOM ration appeared to be

detrimental, indicating that the factor or factors contributing to the poor performance of PSOM protein could not be overcome by increasing the level of protein from PSOM alone.

TABLE 2

Summary of average daily gain, daily feed and feed required per pound of repletion gain for swine

TYPE OF RATION	PROTEIN LEVEL	PROTEIN SOURCE	TREATMENT NUMBER	DAILY GAIN	DAILY FEED	FEED/ GAIN
	%			*lbs.*	*lbs.*	*lbs.*
Semi-purified	12	SBOM	1	0.93	3.26	3.50
		PSOM	2	0.40	2.20	5.50
	16	SBOM	3	1.18	2.44	2.07
		PSOM	4	0.38	2.36	6.20
Corn	12	SBOM	5	0.98	2.39	2.44
		PSOM	6	0.55	2.36	4.29
	16	SBOM	7	1.60	3.63	2.27
		PSOM	8	0.48	1.26	2.62
		Main comparisons:				
	Type of ration					
	Semi-purified			0.72	2.56	4.32
	Corn			0.90 [1]	2.41	2.91 [2]
	Protein level					
	12%			0.72	2.55	3.93
	16%			0.91 [1]	2.42	3.29
	Source of protein [3]					
	Soybean oil meal			1.17 [1]	2.93	2.57 [2]
	Pumpkinseed oil meal			0.45	2.04	4.65

[1] Significantly faster gains.

[2] Significantly less feed required per pound of gain.

[3] Source of protein × level of protein interaction significant.

No attempts were made to investigate the reasons for the poor quality of PSOM. The low feeding value may be due to a low biological value of its protein. Performance was only slightly improved by combination with corn protein; however, the two protein sources could well be limiting in the same amino acids. A low digestibility *per se* might exist, which may be aggravated by the high fiber content, since up to 8.8% of crude fiber was contributed to the rations by the PSOM.

Also, processing conditions may have influenced the protein value, although both meals were expeller-processed by the same plant. Toxic or inhibitory factors cannot be excluded, especially as the higher levels of PSOM protein resulted in slightly decreased gains in swine fed both basal rations. However, Nehring ('49) reported that pumpkin seed oil meals were

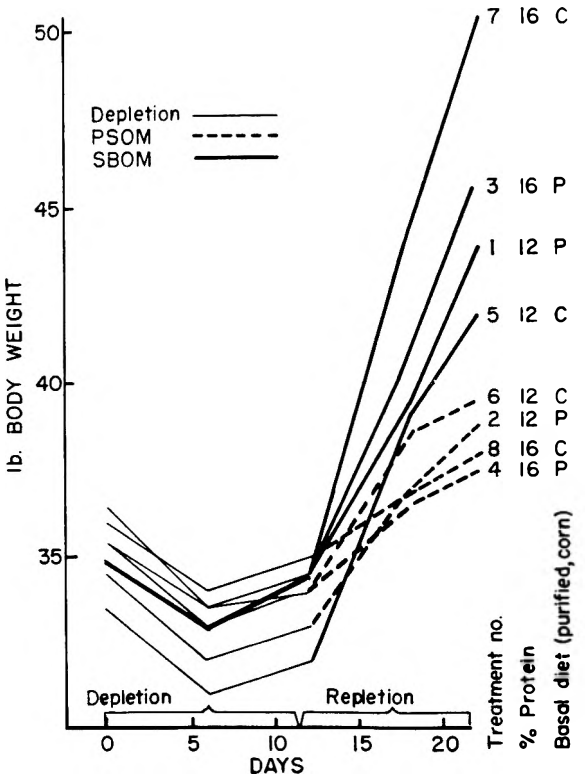

Fig. 2 Body weight changes in swine during depletion and during repletion on two levels of soybean oil meal and pumpkin seed meal protein.

a satisfactory protein source for ruminants and that digestibility of the protein was above 80% even for unhulled meal containing 30% of crude fiber.

An important difference seems to exist in the depletion behavior of rats as compared to swine. Rats of 93 gm body weight responded to the protein-free ration with an immedi-

ate drop in body weight, amounting to about 20% in 8 days. The pigs (35 lbs. body weight) almost maintained their weight over a period of 12 days, and most of them even gained slightly after an initial loss. The different physiological age between the two species and possible differences in the nutritional adequacy of the protein-free rations would not appear to be entirely responsible, since such a consistent weight loss on protein-free rations is known to occur generally in rats regardless of age. Data obtained recently at this station show that even much younger pigs (2 weeks of age and 7 to 10 lbs. body weight) withstand a protein-free diet over a 7-day period of time with little weight loss (Peo et al., '57).

Since a simultaneous comparison with a conventional growth experiment was not conducted, no statement can be made concerning the accuracy of the depletion-repletion technique as a tool for protein evaluation in growing swine. An obvious realimentation, expressed by high growth rate and thereby increased sensitivity to differences in protein quality, should be expected to be the main advantage of such a method. The high daily gain on the 16% protein corn-SBOM ration gives support to the verification of this principle.

SUMMARY

A depletion-repletion technique was employed with growing rats and swine to evalute the protein quality of an expeller-processed pumpkin seed oil meal. Soybean oil meal (expeller-processed) was used as a standard protein source and the two were fed in N-equivalent amounts. Rats and swine responded similarly to each protein source, showing markedly lower performance when fed pumpkin seed meal than when fed soybean meal. Increasing the level of pumpkin seed meal protein in the repletion diet of rats improved gains slightly, but the improved rates of gains were below those observed for the low level of soybean oil meal protein. Increasing the level of pumpkin seed protein in the diet of pigs appeared to depress gains slightly and increase feed required per pound of gain.

A marked difference was observed between the rat and the pig in their response to a protein-free diet, which was otherwise nutritionally well balanced. The growing pigs (35 lbs. body weight) showed little or no weight loss over a depletion period of 12 days, whereas rats (93 gm body weight) lost approximately 20% of their body weight in an 8-day period.

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