DECEMBER 10, 1957

THE JOURNAL OF NUTRITION

VOLUME 63 NUMBER 4

LEROY SHELDON PALMER

Official organ of the American Institute of Nutrition

PUBLISHED MONTHLY BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA 4, PA.

Price, $7.50 per volume, domestic; $8.00 per volume, foreign

Entered as second-class matter January 20, 1934, at the post office at Philadelphia, Pa., under Act of March 3, 1879.

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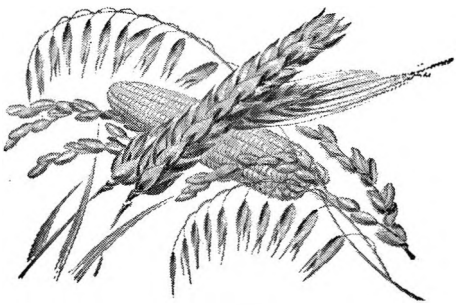

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Protein.....	7.3 gm.	3.1 gm.	4.2 gm.	
Fat.....	5.3 gm.	0.6 gm.	4.7 gm.*	
Carbohydrate	32.2 gm.	22 gm.	6.0 gm.	4.2 gm.
Calcium.....	0.169 gm.	0.025 gm.	0.144 gm.	
Iron.....	1.5 mg.	1.4 mg.	0.1 mg.	
Vitamin A....	195 I.U.	—	195 I.U.	
Thiamine....	0.16 mg.	0.12 mg.	0.04 mg.	
Riboflavin...	0.25 mg.	0.04 mg.	0.21 mg.	
Niacin.....	1.4 mg.	1.3 mg.	0.1 mg.	
Ascorbic Acid	1.5 mg.	—	1.5 mg.	
Cholesterol..	16.4 mg.	0	16.4 mg.*	

*Nonfat (skim) milk, 4 oz., reduces the Fat value to 0.1 gm. and the Cholesterol value to 0.35 mg.

**Based on composite average of breakfast cereals on dry weight basis.

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.

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The Vital Story of VITAMIN C

by Science Writer

What does vitamin C do for human beings?

Medical research shows that it is essential in maintaining healthy bony tissue and for the formation of collagen which is the main supportive protein of skin, tendon, bone, cartilage and connective tissue. It is needed for vascular function and tissue respiration and in promoting the healing of wounds. In lactating women it has great importance in providing the infant with a sufficient amount of ascorbic acid to prevent scurvy, a disease to which very young babies are susceptible.

Much evidence is available in medical reports to show that a lack of this vital nutritive element can result in impaired health. And in those cases of frank scurvy which still occur, ascorbic acid is the specific medication.

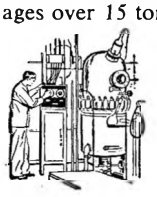
History. The history of vitamin C is fascinating. Scurvy was once the most prevalent disease in Europe. In the 18th Century the antiscorbutic value of fresh vegetables and citrus fruits was recognized.

Isolation. It was not until 1918-25 that almost pure ascorbic acid was concentrated from the lemon by Zilva and its important properties and molecular composition established. In 1927 he concluded that this so-called "reducing factor" and the antiscorbutic principle were closely related. In 1928, Szent-Györgyi, who started investigations in 1921, isolated a strong reducing compound from adrenal glands, oranges, cabbage, terming it "hexuronic acid."

Chemistry. The "reducing factor," "hexuronic acid," and vitamin C were all identified as the same in 1932 by various groups of workers. This was one year after C. G. King established the chemical identification of vitamin C.

Synthesis. In 1933 Reichstein and his colleagues in Switzerland successfully synthesized ascorbic acid. Almost simultaneously in England Haworth and associates accomplished the same. The Reichstein synthesis formed the basis for large scale commercial production by the world-famous firm of Hoffmann-La Roche.

Production. Following these discoveries came the establishment of manufacturing operations so that today daily production by the tons is the rule. The figures are really staggering. The amount produced weekly in the U. S. A. averages over 15 tons, equivalent to that contained in over 200 million oranges. It is being made synthetically at a very low cost which permits widespread use in many food and drug products. One kilo is sufficient to supply 92 people for one year with the minimum daily requirements set by the U. S. Food and Drug Administration.



Vitamin C made by the Roche process is identical chemically and in biological activity with Nature's own product. Because vitamins can be manufactured at a much lower cost than if they were extracted from natural sources they are widely used in dietary supplements.

Ascorbic acid is present in many foods. Nature supplies some with lavish amounts and stints on others. In fact, wide variations* have been found in the vitamin C content in varieties of the same fruit

Deficiencies. Many surveys have shown widespread vitamin C deficiencies in the population. Physicians may call these subclinical, but they are deficiencies nevertheless.

Because of these situations —natural variation and widespread deficiencies—diet experts believe that it is desirable and in the public interest to standardize the vitamin C content of processed fruit and vegetable juices by the addition of enough pure crystalline ascorbic acid to make them consistently dependable sources of this necessary food element. Some of these juices are: tomato, grape, orange, grapefruit, pineapple.

Requirements. The U. S. Food & Drug Administration has set 30 milligrams as the adult minimum daily requirement of ascorbic acid. This daily intake is just about sufficient to prevent recognizable signs of vitamin C deficiencies although a subclinical or an unrecognized deficiency may exist. In view of this, the National Research Council has established 75 milligrams as the recommended daily male adult allowance and 70 mg. daily for female adults, which levels are supported by many controlled nutrition studies. However, the N. R. C. recommends 90 mg. daily for boys between 13 and 15, 100 mg. daily for boys between 16 and 20, and 150 mg. daily for lactating women.

This article is published in the interests of pharmaceutical manufacturers and food processors who make their good products better with essential, health-giving vitamin C. Reprints are freely available. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 1956 Bourdon Street, St. Laurent, P. Q.

*The table shows minimum and maximum levels of ascorbic acid in commercially canned juices. All figures are in milligrams per 100 grams of juice. Data from U. S. Department of Agriculture.

	Min.	*Max.*
Apple juice	0.2	3.6
Grape juice	0.0	4.7
Grapefruit juice	10.0	49.0
Orange juice	9.7	70.0
Pineapple juice	5.4	18.0
Tomato juice	2.5	32.0

PREVENTION OF COPROPHAGY IN THE RAT [1]

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(Received for publication July 10, 1957)

The contributions of coprophagy in rats as a means of making available the nutrients that are synthesized in the lower intestine has remained one of the major nutritional mysteries of our time. The outstanding reviews of Elvehjem in 1948 and Mickelsen in 1956 admirably describe the uncertain qualitative and quantitative importance of coprophagy in supplying the nutrient requirements of the rat. In spite of the generally accepted seriousness of this lack of dietary control, one finds relatively few papers that deal directly with this problem. Geyer and his associates ('47) published some observations on the growth rate of rats in which coprophagy had been prevented by a severe restriction of body movements. Barki et al. ('49) utilized the same technique to study fecal contributions of folic acid and biotin in the rat, and a number of papers have dealt with short-time experiments with rabbits. (Kulwich et al., '53; Thacker and Brandt, '55.)

PROCEDURE

The principal feature of the technique of preventing coprophagy that has been developed in this laboratory is the placement of a plastic cup over the anus and the tail of the rat. This cup is held firmly against the body by a restraining collar that is fastened to the tail. Photographs of the cup and its

[1] This research has been supported in part by a grant from the National Science Foundation.

position on the rat are shown in figure 1. The cups are prepared by cutting off the neck and part of the shoulder of commercially available plastic bottles.[2]

The threaded neck of the plastic bottle may be cut off conveniently with a band saw. This does not leave a good edge unless the cut is low on the shoulder of the bottle. Therefore, it is usually necessary to complete the cut by the use of an

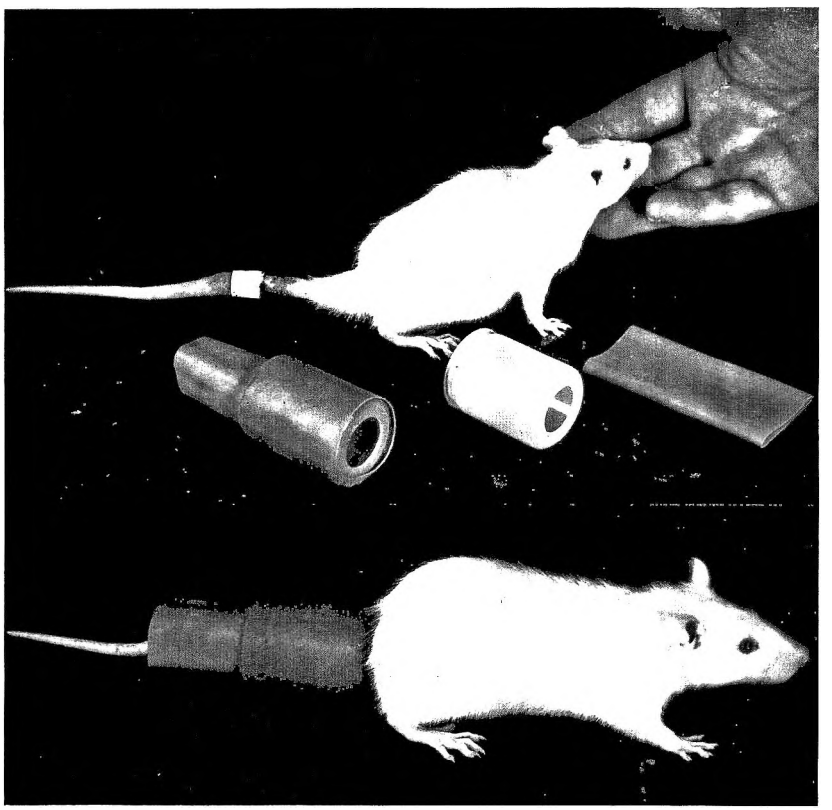

Fig. 1 Component parts of the feces collection cup and its attachment to the rat.

[2] *Plastic cups.* For weanling rats a plastic squeeze-bottle of one-half ounce capacity is used. For larger rats a one-ounce plastic "Boston Round" manufactured by the Wheaton Plastics Company, Mays Landing, New Jersey, was used. For very large rats, a two-ounce "Boston Round" may be adapted.

appropriate drill such as the "Forstner" bit. A small hole of such size as to fit the tail loosely is then drilled in the "bottom" of the plastic bottle. Finally, by means of a wood chisel, the bottom is cut from the edge of the drilled hole to the periphery by two sharp cuts radiating from opposite sides of the "tail" hole. A strip of thin metal [3] just wide enough to cover the plastic cup is then wrapped around with about one-half inch overlap and is held in place by a small piece of Scotch tape. The metal band inhibits partially the rat's insatiable appetite for the plastic cup. A rubber sleeve [4] approximately one inch longer than the cup is slipped over the cup with the excess protruding from the "tail-hole" end of the cup. This excess length of rubber is then folded back over itself so as to leave the "tail-hole" end of the cup exposed. The rubber sleeve helps to hold the metal band in place and the protruding end covers the felt collar that is attached to the tail (see below) and prevents the rat from chewing it off.

The approximate position that the restraining collar will occupy is ascertained and an adhesive [5] is applied to this region of the tail. The adhesive is permitted to dry until it has become tacky. This requires about 10 minutes. The procedure normally followed in this laboratory is to paint the adhesive on the tails of 15 to 20 rats and then apply the cups in the same order. A strip of felt [6] that is just a little over one-half inch wide is cut. For older rats or for rats that have developed sore tails, a wider strip is used. A piece of one-half-inch adhesive tape [7] about three inches long is attached to a short piece of the felt. The felt must be long enough to wrap once around the tail with a very small gap left between the ends. It

[3] *Thin Metal.* Brass shim metal, 0.004 inch thickness.

[4] *Rubber sleeve.* Conventional Gooch rubber tubing "flat" diameter, 1, 1¼ or 1½/inch depending upon the size of the cups.

[5] *Skin adhesive.* With young rats either "Tuf-Skin" or Q.D.A., manufactured by the Cramer Chemical Company, Gardner, Kansas has been found satisfactory. For old rats, "Branding Cement", manufactured by the Nebraska Salesbook Company, Lincoln, Nebraska, has been found fairly satisfactory.

[6] *Felt collar.* One-sixteenth-inch thick felt, purchased from Western Felt Works 4029 – 4137 W. Odgen Ave., Chicago 23, Ill.

[7] *Adhesive tape.* Johnson and Johnson waterproof one-half inch width tape.

must not overlap, nor the ends meet. The cup is slipped over the tail and held firmly against the rump of the rat and the felt collar fastened in place with the aid of the skin adhesive and the short length of adhesive tape. Considerable practice is necessary to establish the correct tension to use in applying the felt collar, so that it does not slip off the tail and yet is not so tight as to damage the tail. The rubber tubing is then unfolded from the cup so that it protrudes over the collar and prevents the rat from chewing it. The tail hole must be of such a size that the rat cannot force this opening down over the felt collar. On the other hand, by squeezing the cup by applying pressure at the opposite edges where the slits were cut, the "tail-hole" opening should enlarge so that the cup can slip over the felt collar in either direction. This facilitates emptying the cup without removing the collar. Approximately once each week the felt collar must be removed and a new one attached. The tails must be examined each day to make sure that excessive pressure is not being applied. Examination of the paper under the wire-bottom cages provides evidence of the adequacy of the cups in catching all fecal pellets. When female rats are used, care must be excerised to obtain the right size "large" opening so that undue pressure against the vagina is avoided. Rats chew the rubber and the cups and it is necessary to replace them continuously. Also, the size of the cups and the size of the "tail-holes" must be enlarged as the rats grow.

RESULTS

Amount of feces consumed by the rat. Although it has been recognized by investigators that the rat will ingest some fecal pellets even though maintained on raised wire screens, no estimate of the quantity of feces thus consumed has been found in the literature. The rabbit has been studied more extensively, but since clearing of the intestinal tract of the rabbit is a very slow process, it is probable that estimates of the extent of coprophagy in this species are subject to large

errors. Nevertheless, figures of approximately 50 to 80% have been presented as estimates of the proportion of excreted feces that are eaten.

Utilizing the present technique for the prevention of coprophagy in the rat that is maintained on a highly purified diet without any added bulk material, the total fecal excretion in the coprophagy-prevented rat (dry weight of feces in the fecal collection cup) is invariably less than in the rat that can eat his feces (dry weight of feces falling through the screen floor of the cages, table 1, experiment A). In the rat in which coprophagy is permitted, feces are recycling and if fecal

TABLE 1

Fecal consumption by rats

DAYS AFTER TAIL CUPS REMOVED	Diet	COPROPHAGY PERMITTED			COPROPHAGY PREVENTED			FECES INGESTED
		No. of rats	Av. Wt.	Feces excreted [1]	No. of rats	Av. Wt.	Feces excreted [1]	
			gm	*gm*		*gm*	*gm*	%
			Experiment A					
	Complete	10	209	1.00	10	189	0.83	...
			Experiment B					
	Complete, low fat				10	202	3.44	75
					9	190	3.45	45
	B_{12} deficient				10	174	...	100
			Experiment C					
	Complete	5	274	0.36	5	270	0.79	54
	Complete	5	284	0.39	5	282	0.67	42
	[2]	3	320	0.49	4	314	1.03	52
	Chow [3]	5	349	1.35	5	340	3.81	65
			Experiment D					
1	Complete	4	286	0.68	4	290	0.76	11
7	Complete	4	...	0.51	4	...	0.72	29
19	Complete	4	...	0.20	4	...	0.58	65

[1] Weight of feces air-dried at 100°C. except in experiment B, in which the weight of fresh feces is given.

[2] A purified diet, complete in all known nutrients but containing only 1% fat, and in addition to normal constituents contained 3% ground cellophane and 3% succinylsulfathiazole.

[3] GLF Big Red Dog Food.

excretion were less than in the rat in which coprophagy is prevented, the difference should reflect increased digestibility of food residues and not be a measure of fecal consumption. Since the diets fed in the experiment being discussed had no added undigestible matter, the heavier weight of fecal dry matter in the coprophagic rat may reflect an increased desquamation or bacterial production in the intestinal tract due to the presence of ingested feces. The coprophagic rats were eating about 10% more food and this, too, may have contributed somewhat to the increased fecal output. In any event, measurement of feces under such conditions cannot be used to calculate the extent of fecal consumption.

A second type of measurement of fecal consumption is illustrated in table 1 under experimental design B. In this study rats that were prevented from eating feces by the presence of the fecal collection cups were given access to their feces by placing each day's collection in a separate feeder jar in the cage. The fresh weight of feces that was eaten was then measured each day. In three such experiments 45 to 100% of the feces were eaten. The existence of a mild dietary deficiency in the form of a lack of vitamin B_{12} appeared to increase fecal consumption.

The third type of study (experiment C) was inteneded to give a more accurate measurement of the extent of fecal consumption in the rat that is maintained on wire screen for the purpose of inhibiting fecal ingestion—the usual practice in modern nutrition research. First the approximate time of food passage through the intestinal tract was estimated by giving rats in which coprophagy was prevented a water suspension of carmine by stomach tube and watching for the appearance of color in the feces. Since defecation in the rat is most prominent during the night hours, and during the day is relatively sparse, an accurate timing of food passage is difficult. Nevertheless, it was found that the carmine marker given at about 3 P.M. was beginning to appear in the excreted feces of all rats by 9 A.M. the following day. This was determined only for rats that had been maintained on a purified

diet without added bulk. On this basis tail cups were attached to rats, usually 10 at a time, in metabolism cages with paper towels underneath in place of funnels, and left from approximately 3 P.M. to at least 9 A.M. the following day. More frequently, a full 24 hours was allowed. At the end of this time any feces that are being produced represent excreta that had not been contributed to by recyclized feces. The fecal collection cups were then emptied and replaced on half of the rats. The remaining half were permitted normal access to their feces. At the end of another overnight period (usually 3 P.M. to 9 A.M.) feces in the cups or on the papers under the coprophagic rats were collected and dried at 100°C. for 24 hours and then weighed. The difference in weight between the two groups was taken as a measure of the amount eaten, and from these figures the percentage ingested was calculated. Table 1 shows that in two experiments with purified diets 42 and 52% of the excreted feces were eaten. In one study with a diet containing 2% of succinylsulfathiazole and 3% of ground cellophane, 52% of feces were eaten, and in one study with a commercial dog chow, 65% ingestion was found.

Eight male rats that had been fed a normal purified diet and had been prevented from coprophagy by means of tail cups continuously for 4 weeks postweaning were selected for experiment D. These rats were divided into two groups and placed in metabolism cages as in the previously described studies. After an overnight holding period tail cups were removed from 4 of the rats and the feces were collected over the next 24-hour period. Dry weights of feces showed that only 11% of feces were consumed by the rats in which coprophagy was permitted. This was probably within experiment error. The cups were removed from the remaining rats and they were held for 6 additional days. On repeating the measurement of fecal consumption at the end of this time 29% fecal consumption was found. Cups were again removed and the rats were held for an additional 12 days. After this period, during which coprophagy was permitted, a measurement of fecal consump-

tion indicated 65% ingestion of excreted feces. During all of these studies the rats were maintained on raised wire screens.

DISCUSSION

The possibility of coprophagy contributing to the nutrition of the nonruminant animal needs no further emphasis. Many authors have commented on how this act could influence the interpretation of experimental results. In view of the assumed importance of coprophagy, it is rather surprising that so little effort has been given to its control. The use of collars or stanchions to prevent coprophagy in rabbits has led to some appreciation of the importance of coprophagy in this species. In the rat, the two studies that have been found in the literature prevented coprophagy by practically complete immobilization in a tubular cage. Geyer et al. ('47) and Barki et al. ('49).

The absolute quantity of feces ingested as measured by the technique described in this paper (experiment C) may be in error. Since it is necessary to prevent coprophagy for approximately 24 hours prior to the period in which coprophagy is permitted, the rats may have developed an appetite in excess of that which is normal. Therefore, the results presented here may be too high. Nevertheless, the rat can and does consume a large proportion of his fecal output even though being maintained on raised wire screens. This makes it a little difficult to understand the remarkable differences in growth rates that early investigators (Steenbock et al. '23) found with rats that were raised on litter as compared to rats on wire screens. One possible explanation is that an important part of vitamin synthesis occurs after the feces are excreted and thus time for additional synthesis would be permitted if the feces were dropped and then consumed at a later time.

It would seem that the rat must learn to eat his feces. In experiment D no feces were consumed the first day after removal of the tail cup which had been attached continuously for 4 weeks. Even 7 days after removal of the cup fecal

consumption was only about half of normal. However, coprophagy to the full extent developed in these rats within 19 days.

Measurements of the extent of coprophagy in the rabbit have utilized unabsorbed cations (Eden, '40), isotopic tracer techniques and measurement of the weight of fecal output (Thacker and Brandt, '55). Since the intestinal tract of the rabbit is practically impossible to clear of all ingested material, weight measurements alone are probably inadquate. It is surprising that the general order of magnitude for fecal consumption in the rabbit corresponds so closely with the calculation reported here for the rat. It seems likely that most nonruminant species have a voracious appetite for feces. This practice is so normal to their nutritional behavior that the cecum and large intestine should rightfully be considered as functionally positioned ahead of the absorptive region of the intestinal tract in a manner similar to the position of the rumen rather than at its distal end as shown anatomically.

Man's behavioral development precudes his indulgence in coprophagy with the exception of occasional manifestations in infants and in insane individuals. Therefore, coprophagy marks one of the major differences affecting nutrition in experimental animals and in man. Obviously many experimental results may be subject to erroneous interpretation unless this important nutritional factor is controlled.

SUMMARY

A technique has been described for the total prevention of coprophagy in the rat. The procedure can be used with weanling rats where growth curves are being obtained, or with older rats in which excreta are being collected for metabolism studies. Measurement of the extent to which rats maintained on raised wire screens eat their feces indicates that 50 to 65% of the excreted feces are normally ingested. In the presence of a nutritional deficiency, fecal consumption may be even greater.

LITERATURE CITED

BARKI, V. H., P. H. DERSE, R. A. COLLINS, E. B. HART AND C. A. ELVEHJEM 1949 The influence of coprophagy on the biotin and folic acid requirements of the rat. J. Nutrition, *37:* 443.

EDEN, A. 1940 Coprophagy in the rabbit. Nature, *145:* 36.

ELVEHJEM, C. A. 1948 Nutritional significance of the intestinal flora. Federation Proc., *7:* 410.

GEYER, R. P., B. R. GEYER, P. H. DERSE, T. ZINKIN, C. A. ELVEHJEM AND E. B. HART 1947 Growth studies with rats kept under conditions which prevented coprophagy. J. Nutrition, *33:* 129.

KULWICH, R., L. STRUGLIA AND P. B. PEARSON 1953 The effect of coprophagy on the excretion of B vitamins by the rabbit. Ibid., *49:* 639.

MICKELSEN, O. 1956 Intestinal synthesis of vitamins in the nonruminant. Vitamins and Hormones, Academic Press, *14:* 1.

STEENBOCK, H., M. T. SELL AND E. M. NELSON 1923 Vitamine B: I. A modified technique in the use of the rat for the determination of vitamine B. J. Biol. Chem., *55:* 399.

THACKER, E. J., AND C. S. BRANDT 1955 Coprophagy in the rabbit. J. Nutrition, *55:* 375.

A CHICK-GROWTH FACTOR IN EGG YOLK

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(Received for publication July 8, 1957)

Denton et al. ('54) were the first to report observations on an unidentified chick growth factor in egg yolk. The addition of yolk to a chick ration resulted in a weight gain 18% greater than that obtained with a basal diet which was adequately fortified with the known required nutrients including vitamin B_{12}. The growth response exhibited by yolk was also obtained in the presence of an adequate supplement of fish solubles and lard. This effect appeared to be additive as chicks fed a combination of fish solubles, fat and yolk grew 32% more rapidly than did those in the control group. These results indicate that yolk contains a growth factor not identical with the fish factor. Arscott ('56) confirmed these findings in his report by stating that egg yolk appears to contain at least one additional growth factor other than the fish factor. However, Hooper et al. ('56) suggested that, according to their data, egg yolk owes its major growth-promoting ability to the fish factor contained therein.

The studies contained in this report were designed to investigate further the relationship between the fish and the yolk factor(s), and to present some of the physical and chemical properties of the yolk factor(s).

METHODS

Day-old New Hampshire x Silver Cornish sex-linked male chicks were used in all of the studies. These chicks were the

progeny of hens maintained on deep litter and fed a complete breeder ration containing fish and meat meals. The chicks were distributed into uniform lots of 10 each on the basis of body weight, wing-banded, maintained in electrically-heated batteries, and fed the basal diet alone, or the basal diet

TABLE I

Composition of basal diet

INGREDIENT	AMOUNT
	%
Ground yellow corn	57.3
Soybean meal	32.0
Alfalfa meal	5.0
Butyl fermentation solubles[1]	0.6
Steamed bone meal	3.0
Ground limestone	1.0
Iodized salt + Manganese[2]	0.5
A–D feeding oil[3]	0.3
Vitamin B_{12} supplement[4]	0.1
Choline supplements[5]	0.1
DL-Methionine	0.1
	100.0
	mg/100 gm
3-Nitro 4-hydroxyphenyl arsonic acid	5.0
Chlortetracycline	2.0
Folic acid	0.2

[1] Contains 500 μg riboflavin per gram.
[2] Made up of 96 parts iodized NaCl and 4 parts $MnSO_4 \cdot H_2O$.
[3] Vitamin D, 400 I. C. U. and 1200 U. S. P. units vitamin A.
[4] Contains 3 mg vitamin B_{12} per pound.
[5] Contains 98,415 mg choline per pound.

containing the various supplements as listed in the tables. Feed and water were supplied ad libitum. At the end of the 4-week experimental period, the birds were weighed individually and the weights recorded.

The composition of the basal diet used in experiments 1 through 9 is shown in table 1. The diet was modified to exclude alfalfa meal and butyl fermentation solubles for

use in experiments 10 and 11 (table 4), and again modified to include 4% fish solubles for use in experiments 12 and 13, and 4% fish solubles plus 5% stabilized lard for use in experiment 14 (table 4). The fluid yolk was obtained by separating the yolk from eggs laid by hens maintained on a complete laying ration containing fish and meat meals. The dried yolk used in these studies was a feed grade product obtained from the manufacturers.[1] The fish solubles, where used, were sardine solubles. The fish-solubles concentrate (20 to 30 times the potency of fish solubles) used in experiment 7 (table 3) was prepared in this laboratory (Menge, Lillie and Denton [2]). Oleic acid (U. S. P.) was used in experiments 1, 2 and 3; and technical grade linoleic acid was used in experiments 4 and 5, (table 2).

The yolk extracts used in experiments 8 (table 3) and 14 (table 4) were prepared by agitating 1500 gms of dried yolk with 3 l of diethyl ether in a large covered jar until thoroughly mixed. After allowing several hours of settling, the supernatant solution was removed by filtration and the procedure repeated three times with 3-l volumes of petroleum ether (b. p. 60 to 70°C), and once again with 3 l of diethyl ether. The residue was air-dried at room temperature. The supernatant solutions were combined and concentrated to a convenient volume at 25°C under reduced pressure in an atmosphere of nitrogen. The ethanolic extracts of dried yolk used in experiments 9, 11, 12 and 13 were prepared by refluxing 1500 gms of yolk with 6 l of ethanol for 15 minutes. The mixture was then filtered and the residue returned to the flask with another 6-l portion of ethanol. The above procedure was repeated three times using fresh ethanol for each reflux. The residue was air-dried at room temperature after extraction and filtration. The supernatant solutions were combined and concentrated to 1 l at 25°C under reduced pressure in an atmosphere of nitrogen.

[1] Through the courtesy of Armour and Company, Research Division, Chicago 9, Illinois, and Henningsen, Inc., Central Laboratories, Springfield, Missouri.

[2] Unpublished data.

Saponification (hot) of an ethanolic extract of yolk was accomplished by refluxing the extract in 10% ethanolic KOH for 4 hours, and then separating the saponifiable from the non-saponifiable portion by the usual procedures. These preparations were used in experiment 11 (table 4). The cold saponification was obtained by stirring a yolk extract (ether-petroleum ether, b. p. 60 to 70°C) in 10% ethanolic KOH at room temperature for 6 hours. The mixture was then placed in the refrigerator for several days before separation of the saponifiable from the non-saponifiable. These fractions were used in experiment 14 (table 4). An ethanolic extract of yolk was subjected to ultra-violet irradiation (3660 to 3900 A) for one-half hour. The extract was placed in a shallow porcelain tray directly under the lamp and stirred continuously. This fraction was used in experiment 12 (table 4). The lecithin used in experiment 12 (table 4) was prepared from dried yolk by the method of Levene and Rolf ('27) with modifications by Maltaner ('30).

The phospholipids in the ethanolic extracts of yolk were separated by precipitation in 5 volumes of acetone containing 2 ml of ethanol saturated with $MgCl_2$ per volume of acetone. The precipitate was washed several times with acetone and then dissolved in a minimum amount of ether. The acetone precipitation and washings described above were then repeated. The supernatant solutions were combined and the acetone was removed under reduced pressure and in an atmosphere of nitrogen. The precipitate was redissolved in ether and all traces of acetone were removed as described above. These preparations were used in experiment 13 (table 4).

RESULTS AND DISCUSSION

According to results obtained by Riemenschneider et al. ('38), yolk fat contains approximately 50% oleic acid and 11% linoleic acid. Since these acids were readily available, it appeared advisable to determine their effect on chick growth in relation to the growth response obtained by

feeding fresh or dried yolk. The results of these trials are presented in table 2. It is apparent that the only consistent improvement in growth rate was obtained in the groups fed either the fresh or the dried yolk. The response obtained with 4% oleic acid and 2% linoleic acid in the presence of yolk was not considered to be significant. These results

TABLE 2

Growth response of chicks to fresh and dried yolk, oleic acid and linoleic acid.

SUPPLEMENT	AVERAGE 4-WEEK WEIGHTS				
	Experiment No.				
	1	2	3	4	5
%	*gm*	*gm*	*gm*	*gm*	*gm*
None	358	334	385	334	382
Dried yolk, 12	...	...	...	373	430
Fresh yolk, 20	401	373	450	...	...
Oleic acid, 2	...	...	394	...	...
Oleic acid, 4	353	...	...	...	...
Oleic acid, 2 and yolk[1]	...	372	442	...	...
Oleic acid, 4 and yolk[1]	439	...	...	...	...
Linoleic acid, 2	...	...	...	...	393
Linoleic acid, 4	...	...	...	...	380
Linoleic acid, 2 and yolk[2]	...	...	...	397	...
Linoleic acid,[4] and yolk[2]	...	...	...	...	434

[1] Fresh yolk, 20%.
[2] Dried yolk, 12%.

suggest that the growth response exhibited by yolk is not due to the presence of the two unsaturated fatty acids under investigation.

The growth stimulatory properties of fish solubles have been under investigation in this laboratory for a number of years (Lillie et al., '53, Menge et al., '53). These observations have shown conclusively that a diet supplemented with 4% fish solubles contains an adequate quantity of the fish factor. Consequently, the data presented in experiments 6, 7 and 8 (table 3) show clearly that the yolk factor is not identical with the fish factor. A growth response to fish solubles is noted in the three experiments cited. The addition of fat in

experiment 8 did not influence growth. Dried or fresh yolk fed alone in experiments 6 and 7, respectively, stimulated growth of chicks in excess of that observed with fish solubles. A fish solubles concentrate (experiment 7) supported a growth rate comparable to that obtained with the original fish solubles supplement. However, when the concentrate

TABLE 3

Chick growth response to fish solubles, stabilized lard, dried yolk and extracts of dried yolk.

EXPERIMENT NO.	REPLICATIONS	GROUP NO.	SUPPLEMENTS	AVERAGE 4-WEEK WEIGHTS
			%	gm.
6	1	1	None	335
		2	Fish solubles, 4	372
		3	Dried yolk, 12	396
		4	As 3 + fish solubles, 4	435
7	1	1	None	342
		2	Fish solubles, 4	380
		3	Fresh yolk, 20	419
		4	Fish solubles conc. $\simeq$ 12%	383
		5	As 4 + fresh yolk, 20	446
8	1	1	None	385
		2	Stabilized lard, 5	383
		3	As 2 + fish solubles, 4	409
		4	As 3 + dried yolk, 16	442
		5	As 3 + yolk extract $\simeq$ 16%	444
		6	As 3 + yolk residue $\simeq$ 16%	417
9	1	1	None	356
		2	Dried yolk, 12	403
		3	Yolk extract $\simeq$ 12%	411
		4	Yolk residue $\simeq$ 12%	363

was fed in combination with fluid yolk, an additive effect on growth was noted. The results obtained in experiments 6 and 8 are comparable, for fresh or dried yolk fed in combination with fish solubles promoted more rapid growth of chicks than did the fish solubles alone.

Furthermore, the fish factor is water soluble (Menge, Lillie and Denton [3]; Menge et al., '53), whereas all of the

[3] See footnote 2 on page 501

extractions and separations of the yolk factor to date indicate that the yolk factor is fat soluble. It is apparent, therefore, that yolk contains a growth stimulatory factor, or factors, not identical with the fish factor. These results are in accord with the work of Denton et al. ('54), and Arscott ('56). The hot ethanol extract used in experiment 8, and the ether-petroleum ether extract used in experiment 9 (table 3) were both active in promoting growth of chicks. The residues were inactive.

According to an earlier report from this laboratory, a growth response to an inorganic substance was obtained with chicks fed the practical type basal diet (table 1) used in the present studies (Menge et al., '56). Therefore, at the start of the yolk extraction procedures, it was feasible to determine whether or not a portion of the growth-promoting principle(s) contained in yolk was due to an inorganic substance. However, the results obtained in experiment 10 (table 4) show that the yolk factor is not inorganic, since ashing dried yolk at 550°C destroyed the growth-promoting action of yolk. There was some speculation that the cholesterol contained in the yolk was acting to overcome the growth-depressing effect of the saponins supplied by the alfalfa meal in the diet. Consequently the ration used in experiments 10 and 11 (table 4) was modified to exclude alfalfa meal. Butyl fermentation solubles was also excluded as a possible source of unidentified growth factors. The basal diet was then fortified by the addition of the following in milligrams per kilogram of diet: riboflavin, 4; niacin, 6; calcium pantothenate, 2; and menadione, 2. It is apparent from the results obtained in experiments 10 and 11 (table 4) that the removal of alfalfa meal from the diets did not affect the growth response to yolk.

According to the results of experiment 11 (table 4), hot saponification of a yolk extract destroyed the yolk factor, whereas cold saponification did not have an effect on the growth-promoting principle (exp. 14, table 4). The effect of ultra-violet irradiation on an extract of yolk was de-

termined in experiment 12 (table 4). Evidently some change had taken place in the yolk solution during the irradiation since the fraction exhibited no growth activity when compared with a similar material not irradiated. Lecithin prepared from a quantity of dried yolk was also inactive (experiment 12, table 4). The results shown in experiment 13 (table 4) indicate that the yolk factor is a non-phospholipid. According to the data shown in experiment 14 (table 4), it

TABLE 4

Growth responses obtained from chicks fed dried yolk and fractions prepared from dried yolk

EXPERIMENT NO.	REPLICATIONS	GROUP NO.	SUPPLEMENTS	AVERAGE 4-WEEK WEIGHTS [1]
			%	gm
10	2	1	None[2]	336
		2	Dried yolk, 12	400
		3	Dried yolk, 12 (ashed 550°C)	356
11	2	1	None[2]	383
		2	Dried yolk, 12	420
		3	Saponifiables ≏ 12% yolk	377
		4	Non-saponifiables ≏ 12% yolk	376
12	1	1	None[3]	387
		2	Dried yolk, 12	445
		3	Yolk extract ≏ 12% yolk	417
		4	As 3 + ultra-violet irradiation	376
		5	Lecithin ≏ 24% yolk	355
13	5	1	None[3]	382
		2	Dried yolk, 12	437
		3	Non-phospholipids ≏ 12% yolk	416
		4	Phospholipids ≏ 12% yolk	388
14	3	1	None[4]	402
		2	Dried yolk, 12	441
		3	Saponifiables (cold) ≏ 12% yolk	408
		4	Non-saponifiables (cold) ≏ 12% yolk	438

[1] Average of replications reported.

[2] Basal diet modified to exclude alfalfa meal and butyl fermentation solubles.

[3] Basal diet contained 4% fish solubles.

[4] Basal diet contained 4% fish solubles and 5% stabilized lard (calorie: protein ratios adjusted).

would appear that the yolk factor is present in the non-saponifiable portion of a yolk extract. The non-saponifiable portion of yolk would include tocopherols, cholesterol, and related sterols, etc. These results are in confirmation of the data shown in the preceding experiment in which the yolk factor was noted to be present in the non-phospholipid portion of a yolk extract. The non-phospholipids would include glycerides, tocopherols, cholesterol, and related sterols. The diets used for the groups in experiment 14 were all adjusted to a calorie: protein ratio of $43 \pm 1.5:1$. This ratio, according to data reported by Donaldson, et al. ('56), is within the range found to be adequate for growth and feed efficiency of chicks to 4 weeks of age. The adjustment in the calorie: protein ratios had no influence on the growth response to yolk. Apparently the growth response to yolk was not due to a more favorable balance of calories and protein.

SUMMARY

Fresh or dried yolk was shown to contain an unidentified chick growth factor (s) not identical with the fish solubles factor. Preliminary fractionation procedures have shown that the active principle is fat soluble since it can be extracted from dried yolk by the common fat solvents. The yolk factor is not identical with oleic acid, linoleic acid, or lecithin. The non-phospholipid and the non-saponifiable portions of yolk extracts contain the growth factor. The yolk factor is destroyed by hot but not by cold saponification. Adjustments in the calorie : protein ratio of the diet and omission of alfalfa leaf meal (source of saponins) from the ration did not affect the growth response to yolk.

LITERATURE CITED

ARSCOTT, G. H. 1956 Complexity of a chick growth response to egg yolk, animal fat and fish solubles additions to the diet. Poultry Sci., *35:* 338.

DENTON, C. A., R. J. LILLIE AND J. R. SIZEMORE 1954 Effect of egg yolk, fat and fish solubles on growth of chicks. Federation Proc., *13:* 455.

DONALDSON, W. E., G. F. COMBS AND G. L. ROMOSER 1956 Studies on energy levels in poultry rations. I. The effect of calorie : protein ratio of the ration on growth, nutrient utilization and body composition of chicks. Poultry Sci., *35:* 1100.

HOOPER, J. H., H. M. SCOTT AND B. C. JOHNSON 1956 The growth promoting ability of fluid egg yolk. Ibid., *35:* 195.

LEVENE, P. A., AND I. P. ROLF 1927 The preparation and purification of lecithin. J. Biol. Chem., *72:* 587.

LILLIE, R. J., J. R. SIZEMORE AND H. R. BIRD 1953 Unidentified factors in poultry nutrition. 1. Development of chick assay. Poultry Sci., *32:* 855.

MALTANER, F., 1930 A note on the preparation of lecithin. J. Am. Chem. Soc., *52:* 1718.

MENGE, H., C. A. DENTON, J. R. SIZEMORE, R. J. LILLIE AND H. R. BIRD 1953 Unidentified factors in poultry nutrition. 2. Properties and preliminary fractionation of a growth factor in condensed fish solubles. Poultry Sci., *32:* 863.

MENGE, H., R. J. LILLIE, J. R. SIZEMORE AND C. A. DENTON 1956 An unidentified mineral required by the chick. Ibid., *35:* 244.

RIEMENSCHNEIDER, R. W., N. R. ELLIS AND H. W. TITUS 1938 The fat acids in the lecithin and glyceride fractions of egg yolk. J. Biol. Chem., *126:* 255.

THE EFFECTS OF BIOLOGICALLY UNAVAILABLE LYSINE (HEATED CASEIN) ON GROWTH

I. BODY WEIGHT AND BONE GROWTH

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(Received for publication July 30, 1956)

INTRODUCTION

Increasing attention has been devoted to the role of lysine in biologic processes in recent years. Previous studies have shown that feeding lysine-deficient diets to rats results in retardation or arrest of their growth, depending on the severity of the deficiency (Harries et al., '43). This retardation is reversed by adding lysine to the diet (Osborne and Mendel, '14). Morgan ('31) pointed out that the nutritive value of casein is greatly impaired by heating, but that supplementation of the heated casein diet with lysine effectively restores its quality. This deficiency probably results from a decreased rate of liberation of lysine from heated casein in the process of digestion, rather than a destruction of lysine *per se* (Pader et al., '48).

The purpose of this study was to investigate the role of lysine in heated casein diets in the processes of growth, particulary of bone.

EXPERIMENTAL

Female rats of the M 13 strain were mated — three females and one male to each cage. The following morning vaginal smears were taken to check for presence of sperm. Insemi-

[1] Louise C. Ball Fellow in Nutrition.

nated rats were placed in individual cages with screen floors and started on the experimental diets. The day of insemination was counted as day zero.

The experiment consisted of three groups, according to the diet fed: group A, basal diet; group B, basal diet plus lysine; group C, stock diet.[2]

TABLE 1

Basic diet composition

CONSTITUENT	AMOUNT	CONSTITUENT	AMOUNT
Wesson oil	145 gm	Celluflour	20 gm
Dextrose	608 gm	Calcium carbonate	5 gm
Salt mixture # 2 G.B.I. (U.S.P. XIII)	40 gm	Cystine	2 gm
		Histidine [1]	4 gm
Heated casein	180 gm		
Vitamin supplements per kilo:			
Choline cholride	1.0 gm	Thiamine	2.0 gm
Inositol	1.0 gm	Riboflavin	4.0 mg
Para-amino-benzoic acid	0.3 gm	Pryidoxine	5.0 mg
		Folic acid	2.5 mg
Nicotinic acid	0.1 gm	Biotin	0.1 mg
Vitamin K	0.01 gm	Vitamin B_{12} 0.1%	5.0 mg
Ascorbic acid	25.0 mg	Alpha-tocopherol [2]	30.0 mg
Calcium pantothenate	10.0 mg	Vitamin A	15000 IU
Lysine [3]	10.0 gm		

[1] Histidine was added, since it has been reported that in the process of heating histidine was also made temporarily unavailable (Morgan, '31).

[2] During pregnancy the amount of alpha tocopherol was doubled.

[3] Ten grams of lysine per kilo of diet was added to the supplemented control (group B).

In a pilot study, a 4th control group was included, using the same basal diet, but with unheated casein and hence no supplement of histidine. The purpose of this was to check on the adequacy of the components of the basal diet. The commercial stock diet was used as a basis of comparison for normal histology and incremental growth. The basal diet is given in table 1.

[2] Rockland.

TABLE 2

Tabulation of weight [1] data of experimental (A) and control (B and C) animals [2]

GROUP	AGE IN DAYS													
A	28	35	42	49	56	60	63	70	77	84	90	98	115	120
1	28	36	56	74	85	92								
2	28	32	49	66	81	91								
3	29	35	46	63	79	90								
4	29	35	44	59	72	80								
5	28	34	55	66	77	85	94	110	116	125	136	151	170	
6	29	35	40	47	59	65	73	84	95	107	122	144	184	190
7	30	36	50	60	72	76	89	108	115	135	147	170	217	220
8	24	32	43	52	70	79	86	100	112	129	142	148	152	
9	25	33	43	50	61	78	85	98	111	129	143	150	156	160
10	23	30	40	53	73	82	86	100	119	139	151	156	163	170
11	25	31	42	50	68	79	86	99	109	125	143	155		165
12	29	35	48	57	68	74	79	88	93	106	122			
13	28	36	43	51	56	58								
14	29	35	51	61	72	79	88	106	123	145	168	203	239	250
15	28	35	49	54	64	69	78	95	105	116	128			
16	28	34	44	53	61	65								
17	28	36	45	51	58	63	67	80	95	115	134	157	185	195
18	40	44	52	76	101	115	125	148	168	178	192			
19	41	46	58	80	107	120	124	132	151	158	185			
20	28	35	48	57	64	71	78	93	109	130	151	181	212	222
B														
1	28	50	74	99	119	125	130	143	159	172	178	180	190	
2	25	49	68	91	113	128								
3	27	50	68	95	116	128	141	167	186	207	216			
4	28	53	70	84	103	117								
5	29	49	69	91	121	150	165	193	214	238	252			
6	27	49	70	92	114	130	145	168	186	208	215			
7	28	50	69	89	113	128								
8	27	54	74	94	112	122	129	148	154	165	185			
9	34	60	84	101	123	140								
10	40	54	75	94	111	115	121	135	144	154	170	179	189	
11	28	48	67	88	109	123	130	145	157	167	174	182	190	198
12	27	48	70	90	111	122	130	143	160	169	174	184	187	195
13	30	53	69	85	100	105								
14	29	47	57	70	83	95	102	122	146	166	177			
15	30	52	72	82	95	105	110	118	130	138	142	150	155	165
16	29	43	63	76	102	108								
17	29	53	74	89	115	121	129	148	164	178	188			
18	35	56	72	89	108	112	115	130	135	150	160	170	182	194
C														
1	55	84	116	150	172	176	180	194	203	215	219	229	239	242
2	54	82	100	118	133	136								
3	55	82	108	133	145	150								
4	56	83	100	119	135	142	150	167	181	189	200			
5	67	124	159	200	235	253	262	296	316	333	347	354	380	386
6	65	100	130	147	163	169	172	188	195	200	208	218	225	227
7	65	99	131	151	165	172	177	194	204	209	217	225		235
8	67	119	167	211	249	266	273	296	312	326	341	365	388	395
9	64	119	141	187	220	238	258	273	298	307	319	347	375	377
10	57	95	127	167	179	188	201	224	246	259	272	289	310	
11	53	88	97	120	134	144	154	162	173	177	179	185	197	201
12	57	85	112	126	140	152	160	187	211	237	250			
13	50	75	96	128	162	186	197	221	239	251	279	284		293
14	40	64	85	105	127	140	146	162	174	177	187	202		
15	45	74	101	137	170	193	205	227	247	259	265	277	293	
16	48	75	106	140	181	208	216	244	265	280	290			
17	59	78	100	113	128	144	156	173	177	186	188			
18	56	80	124	162	200	222	235	253	274	297	310			

[1] Weights in grams.

[2] The table comprises a representative sample for each group.

Heated casein was prepared according to the method described by Morgan ('31): the casein was placed in aluminum pans in layers about 1/4" thick and heated in an oven for 45 to 60 minutes at 145° to 150° C. Dry heat was used.

Food and water were administered ad libitum. Upon delivery each litter was reduced to a uniform litter size of 8. Litters were weighed at birth and every three days thereafter until weaning, after which weights were recorded once weekly. Animals were weaned at 28 days, after which they were kept on the same diets as their mothers. Individual weight charts were prepared. Each experimental group comprised 20 litters, totalling 160 animals, on which weight charts were based (table 2). Of these, 75 were randomly selected for studies of growth and of ash and matrix of the long bones. Animals from each diet group, but belonging to different litters, were sacrificed on the 21st, 29th, 60th, 90th and 120th days post partum.

The left femur of each animal was fixed in 95% alcohol and mechanically cleaned of all soft tissues. Measurements of femur length and width were taken according to the technique used by Levy et al. ('53). These bones were then submitted to lipid extraction in a Soxhlet for 8 hours in 95% alcohol and 16 hours in ether. They were then dried to constant weight and ashed. Ash and matrix weight and bone ash percentage were calculated.

OBSERVATIONS AND DISCUSSION

The animals of the experimental group (A) did not differ from those of the control groups (B and C) with respect to gestation period, litter size or litter weight at birth. The preweaning weight curves show a linear distribution of increments, although there is a certain amount of divergence near the end of the preweaning period. Both the lysine-supplemented group (B) and the unsupplemented group (A) were lighter in weight than the stock diet controls (C) at weaning (fig. 1).

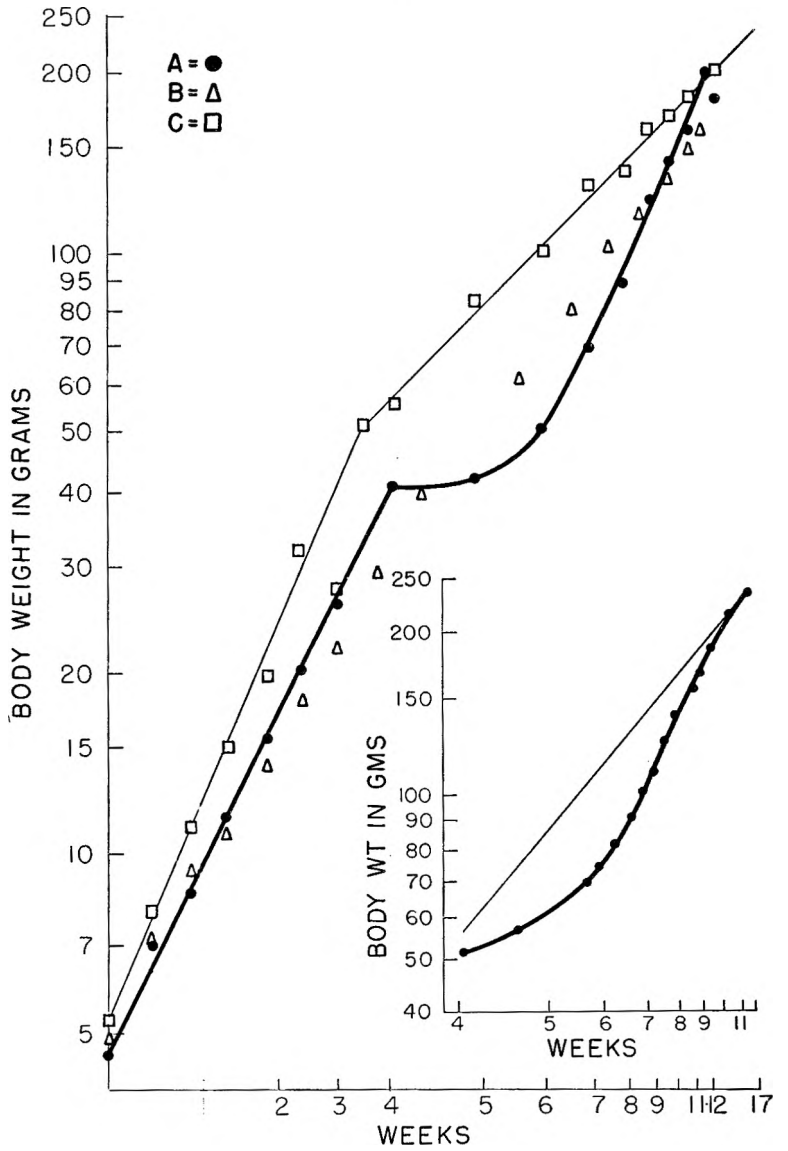

Fig. 1 Growth chart: body weight/time

Main grid: Log W (weight)/reciprocal T (age in weeks) for postweaning growth.

Log W/log T for preweaning growth. (Zucker, et al., '41). Insert: Postweaning growth chart, pilot study.

A = Basal diet B = Basal diet + lysine C = Stock diet

The growth charts (fig. 1) based on body weight show a discontinued allometric relationship in group A. The usually straight line of growth obtained by plotting the log of weight against time on a reciprocal scale (Zucker et al., '41), exhibits a period of weight maintenance or minimal increase in weight between the 4th and the end of the 5th, or beginning of the 6th week. After this, the growth trend changes, a sharp break occurring around the 6th week, with the animals starting to gain weight very markedly. They reach the weight of the normal controls (C) after the 12th week of age. Thus the unsupplemented animals, group A, instead of showing a straight line incremental growth, present a curve with a reversed plateau and a straight line time-weight relationship after the 6th week.

As is evident from figure 1, the lysine-supplemented control group (B) begins on a slightly lower average weight than the unsupplemented group (A), and the slope of the growth curve has a more acute angle than does the curve for the normal control (C). However, the incremental growth shows a completely linear and proportionate relationship.

The fact that the growth pattern of group B is quite different from that of group C (which has been shown by Zucker et al., '41 to be the true population slope for rats), and also that group B starts out on a lower average weight, indicates that heated casein, although supplemented with histidine and lysine, does not support optimal growth. Similar observations have been reported by Harries, Neuberger and Sanger ('43) with supplemented gliadin and zein diets Melnick, Oser and Weiss ('46) stated that "for optimum utilization of food proteins all essential amino acids must not only be available for absorption, but must also be liberated during digestion *in vivo* at rates permitting mutual supplementation." On this basis one might propose an explanation for the observed suboptimal growth produced by diets supplemented with chemically pure amino acids: that there might be a difference in rate of deamination of naturally

occurring and chemically pure amino acids, and thus the efficiency of their utilization might be reduced. Another possibility might be that some other changes occur during the process of heating the casein in addition to the effect on histidine and lysine.[3] Whatever the explanation, the fact that the supplemented animals present a linear incremental growth pattern seems to indicate that no severe imbalance was produced in the diet of these animals.

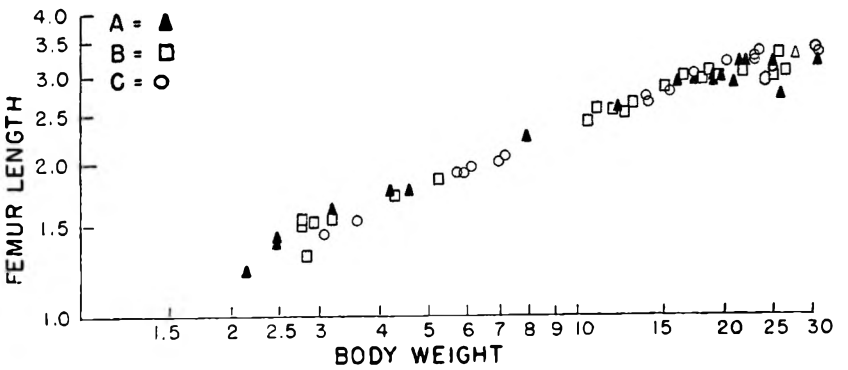

Fig. 2 Femur length plotted against body weight indicating allometric relationship (logarithmic scale).

A = Basal diet
B = Basal diet + lysine
C = Stock diet

The plots of body weight against femur length (fig. 2) exhibit a linear and proportionate slope in all three groups. The experimental measurements, although distributed in a straight line, show a concentration near the lower values of the graph. During the interval between 45 and 80 gm of body weight we find no distribution of group A measurements. This period coincides with the period of growth retardation and weight maintenance. Thereafter the lag is rapidly eliminated, and the findings in the experimental group coincide in distribution with those of the control animals. This

[3] The animals in the pilot study, consuming the same basal diet, but with unheated casein, grew as well as the controls kept on the stock diet.

interval coincides with the time of rapid and spontaneous realimentation, which in the time-weight graph was expressed in a sharp break and sudden rapid gain in weight. The linear relationship in femur length-body weight seems to indicate that any changes induced with the heated casein diet in this study have affected the whole animal with equal intensity.

The ash-matrix ratio (fig. 3) of group A shows an allometric relationship and has the same slope as the control

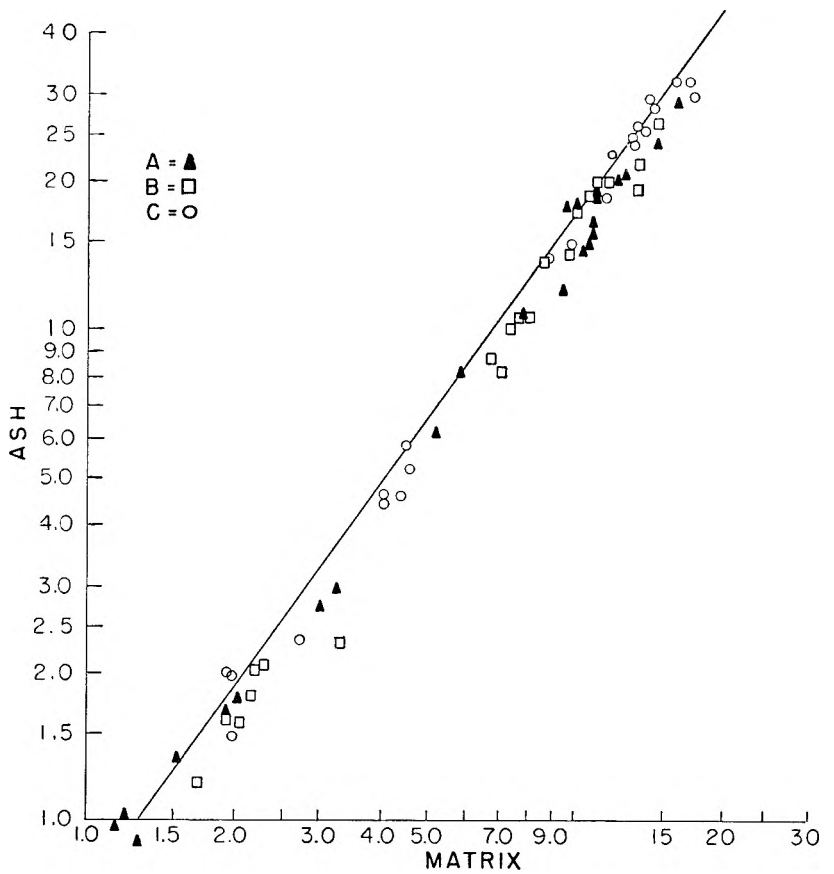

Fig. 3 Femur ash – femur matrix ratio (logarithmic scale)
A = Basal diet
B = Basal diet + lysine
C = Stock diet

groups (B and C), although in the period before realimentation the values fall within the lower ranges of the graph.

The fact that both ash and matrix as related to the body weight (fig. 4) in group A show a proportionate relationship suggests that growth both of the hard and soft tissues has been symmetrically retarded. The same trend of propor-

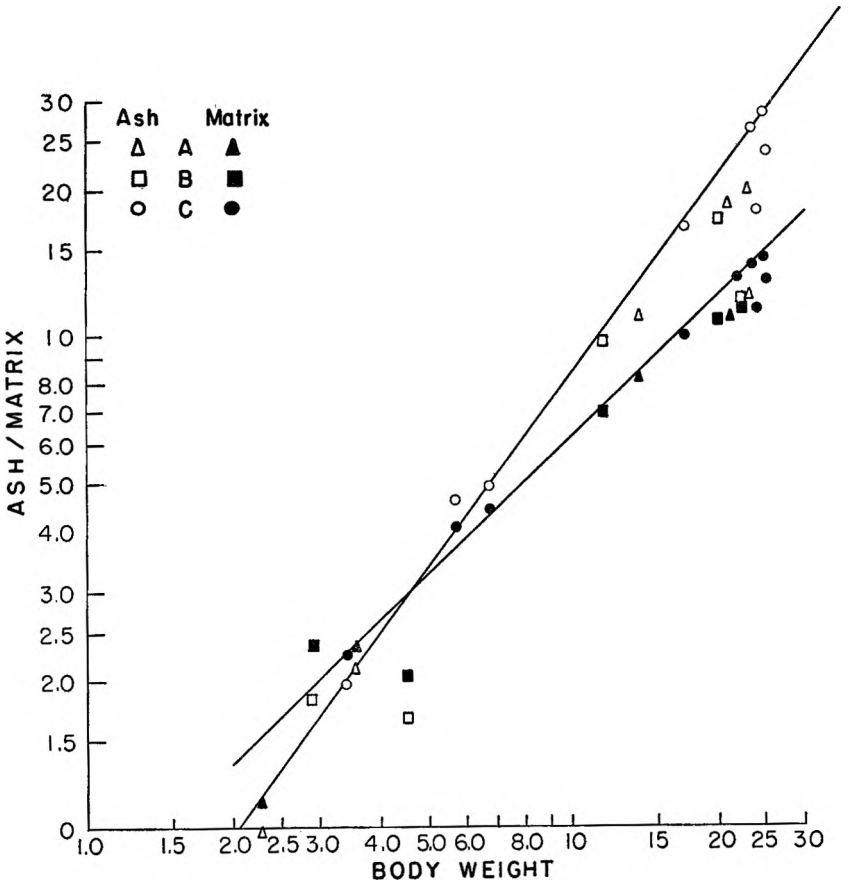

Fig. 4 Femur ash/femur matrix plotted against body weight (logarithmic scale).

A = Basal diet
B = Basal diet + lysine
C = Stock diet

tionality shows up in the relationship of femur ash to dry bone weight (fig. 5), and holds true for all measurements starting from 21 days through 120 days. The increase of bone ash percentage is likewise uniform.

In general it was observed that the animals on the unsupplemented diet (group A) showed the same vitality as the animals in groups B and C. Their appetites did not seem to be reduced, although no food consumption records were kept. Their fur was smooth, and the animals were not prone to infection. Mortality was negligible and was not confined

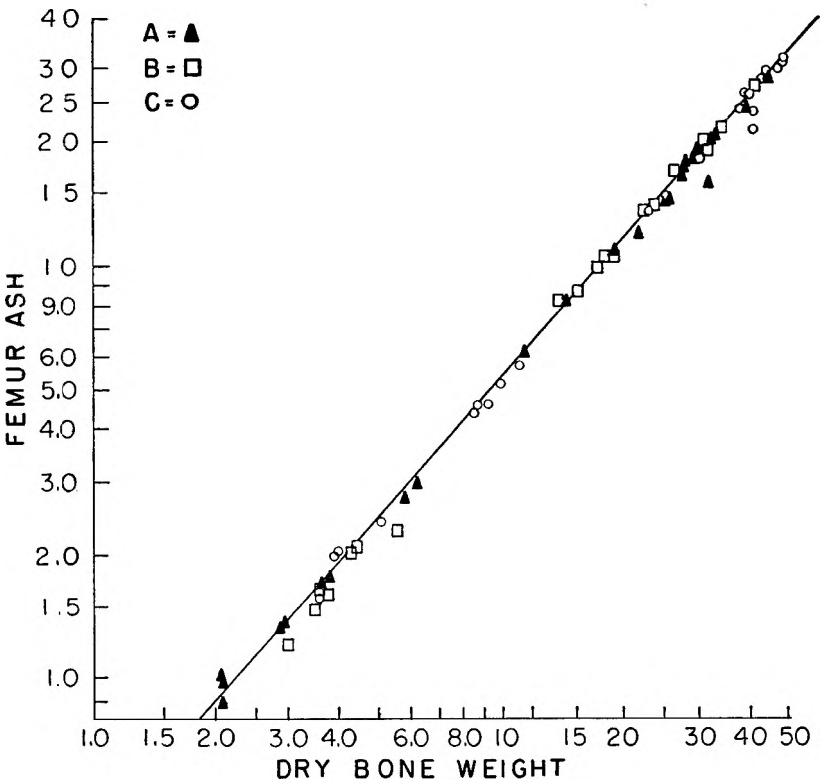

Fig. 5 Femur ash-dry femur ratio (logarithmic scale). Weight in milligrams.

A = Basal diet

B = Basal diet + lysine

C = Stock diet

to group A. In the females of group A the onset of the oestrous cycle was delayed. This contrasts somewhat with the observation of Pearson ('37) who reported a definite suspension of the oestrous cycle in lysine deficiency. This difference may have been due to the deficiency having been more severe in the Pearson animals.

The unsupplemented animals (group A) showed a large amount of subcutaneous fat. Their soft tissues (muscles, tendons, fasciae, and parenchymatous organs) were younger looking — both lighter in color and less tough in consistency — than those of control animals of the same age. The uteri of the unsupplemented animals were smaller than those of both the supplemented and normal controls.

From the above observations, it is evident that we are dealing with two distinct responses to the experimental diet: first, a phase of growth retardation; and second, a phase of spontaneous recovery. The unexpected reversal and return to normal is rather puzzling and difficult to explain. A plausible explanation could be based on the work of Melnick et al. ('46) with heated casein. These authors found that unheated casein contains 8.1% lysine. Heating the casein for 1.5 hours at 150° C, they were able to reclaim 7.9% lysine; no further reduction of the lysine content resulted upon prolonged heating. Furthermore, they found in pancreatic hydrolysis of heated casein *in vitro* that the degree of protein hydrolysis was not greatly affected by the temperature at which the casein was heated. However, the rate of liberation of available lysine from heated casein was greatly reduced when compared to the rate of lysine liberated from unheated casein.

On the basis of the experiments reported in this paper the following tentative explanation is offered: during the preweaning period no drastic weight deflections are observed, except near the end of that period. This might be due to the fact that lysine is supplied in the milk from reserves from the maternal structures. (It was observed that the mothers

in the unsupplemented group lost more weight than those in the control groups during the period of lactation, although no accurate weight charts were kept for the mothers.)

After weaning, during the very active period of growth, these reserves are no longer available; nor is the lysine, though present in the diet, available for protein synthesis. During the period of observed weight maintenance the organism either utilizes the lysine present in the tissues for maintenance of protein balance, or the utilization of the remaining amino acids in the diet is greatly reduced. What we observed until the time of spontaneous realimentation could be attributed to a general protein deficiency. The sudden reversal might possibly be due to the availability of sufficient lysine by this time for adequate amino acid metabolism.

The above explanation might be accepted as a working hypothesis for further investigations.

The persistency with which the proportionate growth retardation and the process of spontaneous recovery repeat themselves in the different methods of analyses of the material, both in the pilot study and in the subsequent experiment, shows that these findings are not a chance occurrence.

SUMMARY

1. A temporary growth cessation, expressed in lowered body weight (allowing of endochondral bone formation) takes place in animals fed adequate diets in which the sole source of protein is heated casein.

2. The imbalance produced by the diet induces a uniform retardation in the formation of both skeletal and soft structures, under the conditions in which this experiment was carried out.

3. The changes occurring during the period of retardation are reversible and self-correcting without supplementation of the diet.

ACKNOWLEDGMENTS

Thanks and appreciation are expressed to Dr. T. F. Zucker and Dr. L. M. Zucker who contributed the animals and made their laboratory facilities available, and for their encouragement and guidance; to Dr. Barnet M. Levy for his untiring assistance in every phase of this study.

LITERATURE CITED

HARRIES, H. A., A. NEUBERGER AND F. SANGER 1943 Lysine deficiency in young rats. Biochem. J., *37:* 508.

LEVY, B. M., R. RUGH, L. LUNIN, N. CHILTON AND M. MOSS 1953 The effect of a single sub-acute x-ray exposure to the fetus on skeletal growth: A quantitive study. J. Morph., *93:* 561.

MELNICK, D., B. L. OSER AND S. WEISS 1946 Rate of enzymic digestion of proteins as a factor in nutrition. Science, *103:* 326.

MORGAN, A. F. 1931 The effect of heat upon the biological value of cereal protein and casein. J. Biol. Chem., *90:* 771.

OSBORNE, T. B., AND L. B. MENDEL 1914 Amino acids in nutrition and growth. Ibid., *17:* 325.

PADER, M. D., D. MELNICK AND B. L. OSER 1948 Factors affecting the availability of lysine in heat processed casein. *172:* 763.

PEARSON, P. B. 1937 The effect of a lysine deficient diet on the oestrous cycle. Am. J. Physiol., *118:* 786.

ZUCKER, T. F., L. HALL, W. YOUNG AND L. ZUCKER 1941 The growth curve of the albino rat in relation to diet. J. Nutrition., *22:* 123.

EFFECT OF FAT IN DIET ON UNSATURATED FATTY ACIDS IN PHOSPHOLIPID, CHOLESTEROL ESTER AND GLYCERIDE FRACTIONS IN SERUM OF DOGS[1]

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(Received for publication June 10, 1957)

The necessity of obtaining more information than is available at present concerning the essential fatty acids is quite apparent not only because of the importance of these fatty acids in infant nutrition but also because of their possible relation to the arteriosclerosis problem. Exact data on the distribution of linoleic and arachidonic acids in the lipid fractions of blood serum as influenced by diet would seem to be particularly significant. As early as 1924 Bloor suggested the importance of cholesterol esters as carriers of unsaturated fatty acids in blood plasma, especially cholesterol linoleate. Kelsey and Longenecker ('41) later reported a high percentage of linoleic acid in the cholesterol esters of beef plasma. However, the above workers did not characterize clearly the unsaturated fatty acids in the phospholipids of the beef, pig or dog plasma which they were studying. Previous studies with dogs and human subjects (Hansen and co-workers, '55, '57; Wiese and co-workers, '51, '53, '54, '56) have shown that the lipid composition of blood serum reflects to a remarkable degree the dietary intake of linoleic acid. Inasmuch as diets deficient in linoleic acid lead to very low serum levels for linoleic and arachidonic acids and result in high levels for a three double-bond fatty acid in young infants as well as in young puppies, it seemed pertinent to determine in which fraction or

[1] Supported in part by a U. S. Department of Agriculture contract sponsored by the Human Nutrition Research Division, Agriculture Research Service.

fractions of the serum lipids these marked alterations occur. Because of the relatively large amount of serum required for separation of sufficient fatty acid for identification in phospholipids, cholesterol esters and glycerides, we have attempted first to characterize the fatty acids in these fractions of dog serum in relation to the dietary intake of fat and linoleic acid. In the course of the study, it has been possible to obtain information concerning the effect of sex and age as well as diet on the distribution of the di-, tri-, tetra-, penta- and hexaenoic acids in the serum of the dogs.

EXPERIMENTAL PROCEDURE

Animals and diets. A total of 22 mongrel dogs have been under study, 12 of which were maintained on a diet low in fat and 10 of which received the same diet except for the isocaloric substitution of fresh lard for 30% of the sucrose calories. The latter animals served as healthy controls. There were an equal number of male and female dogs and an equal number of young (under one year) and adult animals in each group. All the dogs received their respective diets for a minimum of two months before the beginning of the experimental period; 15 were on the same diet from the time of weaning. Both diets contained the same percentage of protein, minerals, vitamins and celluflour and were fed at the same caloric level per kilogram body weight (Hansen and Wiese, '51). The caloric composition of the diets was as follows:

	LOW FAT	CONTROL
Protein	23.6%	23.6%
Powdered skim milk		
Casein		
Carbohydrate	75.5%	46.4%
Powdered skim milk		
Sucrose		
Fat	0.9%	30.0%
Powdered skim milk		
Lard		
Linoleic acid	0.1%	4.0%
Powdered skim milk		
Lard		

Insofar as possible, litter mate animals were used for both the low-fat and control diets. All dogs receiving the low-fat diet showed typical signs of severe fat deficiency, i.e., extensive desquamation, loss of hair, susceptibility to infection, tremulousness and failure to gain weight. Animals in the control group were in excellent condition with soft, pliable skin and hair and showed satisfactory weight gains.

METHODS

Blood samples were taken from all the dogs after an overnight fast. Analyses were made on an average of three blood samples for each animal. Lipid analyses included the amount and distribution of the 2, 3, 4, 5 and 6 double-bond fatty acids in the total, phospholipid, cholesterol ester and glyceride fractions of the serum for both fat-deficient and control animals.

Briefly, the procedure for analysis was as follows: the total lipids were extracted from fasted serum with a 3:1 mixture of ethyl alcohol and ethyl ether (Bloor's mixture) as described by Wiese and Hansen ('53). Usually 15 to 20 ml of serum were used and made up to a volume of 200 ml. Aliquots of the extract equivalent to 3.0 ml of serum were taken for total fatty acids and the remainder of the extract was used for determination of the phospholipid, chloesterol ester and glyceride fatty acids. Separation of these fatty acids was accomplished by precipitation of the phospholipids with acetone (Bloor, '29) and by enzymatic hydrolysis of the glycerides in the acetone soluble fraction with castor bean lipase at pH 4.6 (Kelsey, '39). A schematic representation of steps in the procedure is given in figure 1.

Isomerization of the fatty acids from each fraction was carried out with 11% KOH-ethylene glycol according to the method of Wiese and Hansen ('53). Spectral densities were read on a Beckman D. U. Spectrophotometer. Absorption data were calculated as $E^{1\%}_{1cm}$ and the percent of each unsaturated fatty acid was calculated from standards for linoleic, linolenic, arachidonic and docosahexaenoic acids (Wiese and others, '54).

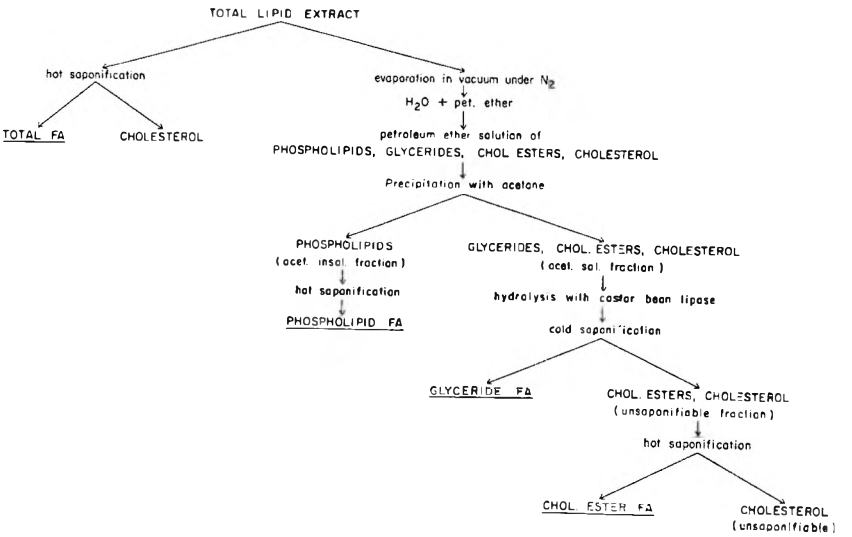

Fig. 1 Schematic representation for separation of serum fatty acids into those derived from phospholipids, glycerides and cholesterol esters. Fatty acids in each fraction are prepared in same manner as total fatty acids (Wiese and Hansen, '53).

RESULTS

Influence of diet on distribution of fatty acids

Total fatty acids. In figure 2 is presented a summary chart showing the mean amount of the total fatty acids which were derived from phospholipids, cholesterol esters and glycerides in blood serum for 10 control and 12 fat-deficient dogs. It is obvious that animals on the low-fat diet definitely had lesser amounts of total fatty acids in the serum than the healthy control animals which received 30% of their calories as fat. This difference is accounted for by decreases in both the phospholipid and cholesterol ester fractions in the low-fat group. There was no real difference in the glyceride fractions.

When the total fatty acids were analyzed for the 2, 3, 4, 5 and 6 double-bond fatty acids, striking differences in composition were noted between the control (C) and low-fat (LF) groups. These results are depicted in figure 3. Whether the data are plotted as absolute amounts (mg%) or on a percent-

age basis, the same relative differences in composition are observed between the animals which received 4% of their calories as linoleic acid and those which received 0.1% of their calories as linoleic acid. There were definite decreases in the di- and

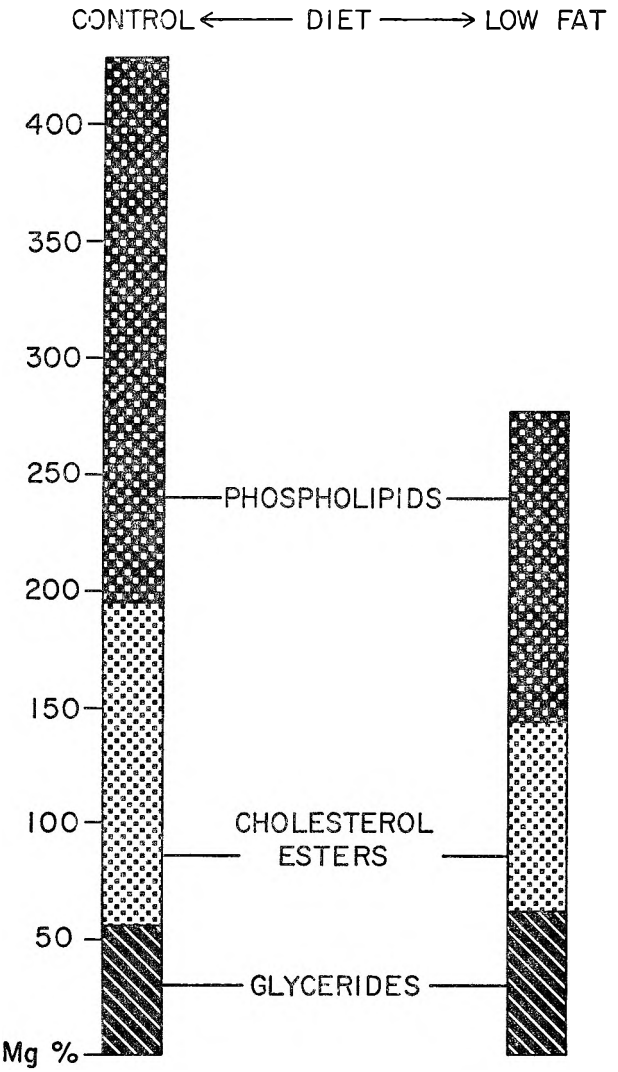

Fig. 2 Fatty acids in phospholipid, cholesterol ester and glyceride fractions (expressed as mg%).

tetraenoic acids and a definite increase in the trienoic acid content of the serum when linoleic acid was extremely low in the diet. The amount of pentaenoic acid has not been calculated because of the lack of a suitable standard. From the absorption data available, however, these values would be small and of the same order of magnitude as for the hexaenoic acids.

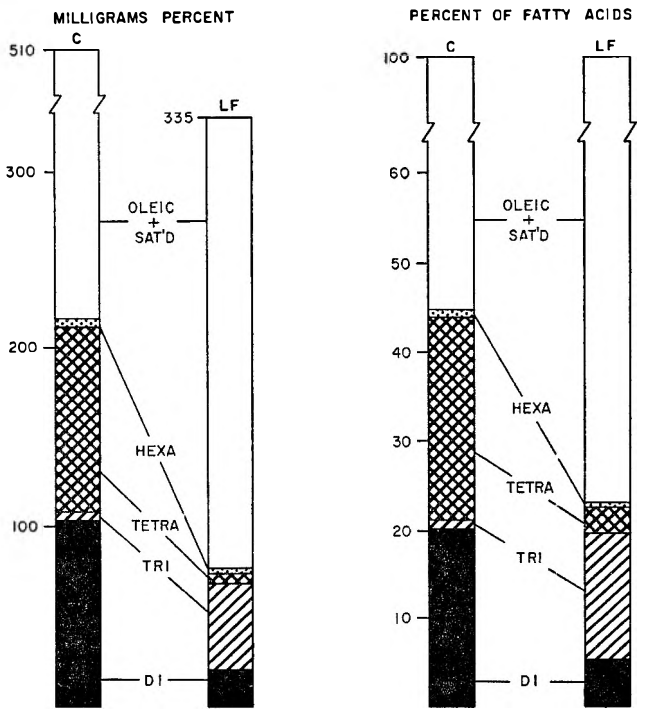

Fig. 3 Distribution of unsaturated fatty acids in total fatty acids of serum for dogs on control and low fat diets.

Phospholipid, cholesterol ester and glyceride fatty acids. Since both the total amount of fat and the distribution of the di-, tri- and tetraenoic acids are influenced by the absence of fat in the diet, we have chosen to depict results for the distribution of these fatty acids as percentage in each fraction for the phospholipids, cholesterol esters and glycerides. These are shown in figure 4. It is evident that in the serum phospholipids

of healthy dogs, the predominant unsaturated fatty acid was tetraenoic acid whereas in the fat-deficient group, trienoic acid predominated. In the cholesterol ester fatty acids, two differences between the groups may be noted: (1) in healthy dogs, these fatty acids were more unsaturated than those derived from the phospholipids, whereas in fat-deficient dogs, the

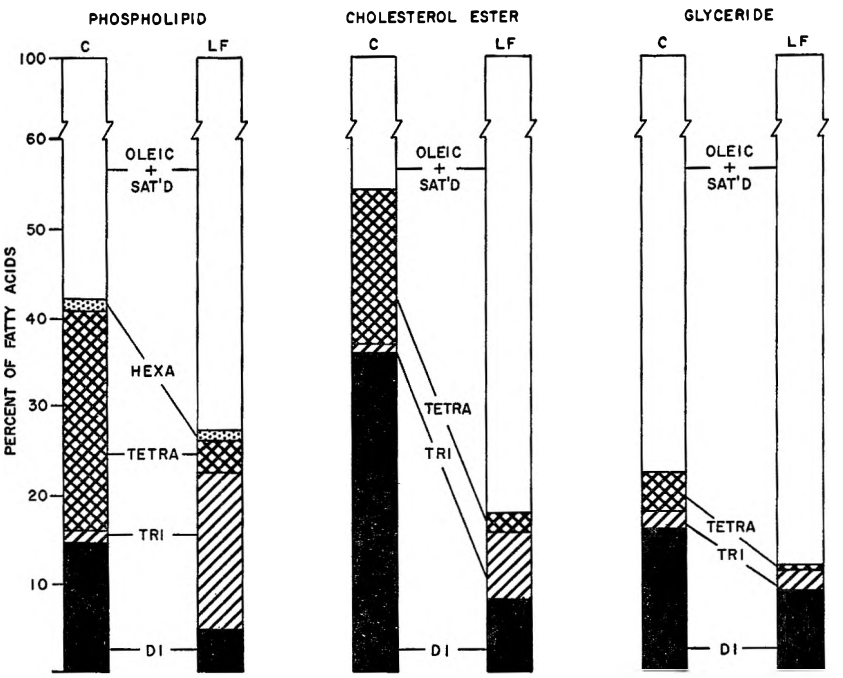

Fig. 4 Distribution of unsaturated fatty acids in phospholipid, cholesterol ester and glyceride fractions of serum for dogs on control and low-fat diets.

cholesterol ester fatty acids were less unsaturated than the phospholipid fatty acids; (2) dienoic acid was the predominant unsaturated fatty acid in the cholesterol ester fraction for the control group, but there were approximately equal quantities of di- and trienoic acids in the low-fat group. Glyceride fatty acids were the least unsaturated in both groups of animals with dienoic acid predominating.

TABLE 1

Statistical summary of serum fatty acids for 10 control dogs and 12 fat-deficient dogs

GROUP	NUMBER		MG%	DIE-NOIC [1]	TRIE-NOIC [1]	TETRAE-NOIC [1]	HEXAE-NOIC [1]
	DOGS	DETERM.					
			Total fatty acids				
Control	10	28					
Mean			510	20.1	0.9	22.5	0.6
SEm			18.6	0.9	0.2	0.9	0.1
Fat-deficient	12	35					
Mean			335	5.0	14.4	2.9	0.3
SEm			13.6	0.2	0.4	0.04	0.1
P [2]			<0.001	<0.001	<0.001	<0.001	<0.001
			Phospholipid fatty acids				
Control	10	30					
Mean			231	14.1	1.3	24.8	1.2
SEm			11.5	0.7	0.3	0.8	0.2
Fat-deficient	12	37					
Mean			127	3.9	17.6	3.8	0.8
SEm			8.0	0.1	0.5	0.2	0.2
P			<0.001	<0.001	<0.001	<0.001	<0.001
			Cholesterol ester fatty acids				
Control	10	27					
Mean			140	35.8	0.7	17.2	0.2
SEm			6.8	1.0	0.2	1.0	0.08
Fat-deficient	12	31					
Mean			85	7.9	7.1	1.8	0.02
SEm			5.2	0.3	0.4	0.4	0.008
P			<0.001	<0.001	<0.001	<0.001	>0.02
			Glyceride fatty acids				
Control	10	25					
Mean			57	15.5	1.6	4.5	0.1
SEm			4.1	1.0	0.2	0.5	0.06
Fat-deficient	12	30					
Mean			60	9.4	2.2	0.3	0.07
SEm			4.8	0.7	0.3	0.1	0.05
P			>0.10	<0.001	>0.02	<0.001	>0.05

[1] As percent of fatty acids in each fraction.

[2] "P" was derived from calculated "t" values (when $N < 30$) using a two tail test of the one tail probability table in Dixon and Massey, '51.

Statistical summary of results. A statistical summary of the data shown in figures 3 and 4 is presented in table 1. The differences between the fatty acids in serums of control and fat-deficient animals which were evident from the graphic presentations are shown to be significantly different in the amount of the total, phospholipid and cholesterol ester fatty acids. The di-, tri- and tetraenoic acids in these same fractions also were significantly different in the two groups of dogs. The hexaenoic acids, although small in amount, were significantly different between the control and fat-deficient groups in the total and phospholipid fractions. The glyceride fatty acids showed significantly less di- and tetraenoic acid in the low-fat group. The sum of the fatty acids derived from phospholipids, cholesterol esters and glycerides is somewhat less than the value for the total fatty acids by direct determination, due to mechanical loss when the lipid extract is washed with water to remove inorganic and nitrogenous contaminants before precipitation of the phospholipids. The calculated distribution of the unsaturated fatty acids from the three fractions is of the same order of magnitude as found for the total fatty acids by direct determination.

Influence of sex on distribution of fatty acids

Inasmuch as there were an equal number of male and female animals in the control and fat-deficient groups, we have analyzed the data with respect to sex. It was evident that for control animals, mean values were essentially the same for male and female dogs. On the other hand, in the fat-deficient group, there was a general trend for female animals to have higher serum levels for some of the fatty acids than male animals. This was true for the amount of fatty acid in the total, phospholipid and cholesterol ester fractions as well as for the trienoic content of these fractions. However, in view of the small differences which could be attributed to sex, a statistical analysis of these data was not made.

TABLE 2

Statistical summary of serum fatty acids with respect to age for control and fat-deficient dogs

GROUP	CONTROL [1]					FAT-DEFICIENT [1]				
	MG%	DI [2]	TRI [2]	TETRA [2]	HEXA [2]	MG%	DI [1]	TRI [2]	TETRA [2]	HEXA [2]
	Total fatty acids					Total fatty acids				
Under 1 yr.										
Mean	484	17.2	1.1	23.6	0.8	290	4.3	13.1	2.8	0.1
SEm	20.4	0.9	0.4	1.4	0.2	14.0	0.2	0.7	0.3	0.1
Over 1 yr.										
Mean	536	23.2	0.6	21.4	0.5	383	5.6	15.5	2.9	0.4
SEm	30.3	1.1	0.2	1.0	0.1	17.0	0.3	0.3	0.3	0.1
P [3]	>0.02	<0.001	>0.10	>0.02	>0.05	<0.001	<0.001	<0.001	>0.50	>0.50
	Phospholipid fatty acids					Phospholipid fatty acids				
Under 1 yr.										
Mean	219	11.9	1.5	26.2	1.6	112	3.3	15.9	3.7	0.5
SEm	16.3	1.2	0.5	1.4	0.4	8.8	0.2	0.7	0.3	0.1
Over 1 yr.										
Mean	241	16.0	1.0	23.5	0.9	142	4.4	19.2	3.9	1.1
SEm	17.8	0.8	0.2	0.9	0.1	12.2	0.1	0.4	0.2	0.3
P	<0.01	<0.001	>0.10	>0.01	>0.05	<0.01	<0.001	<0.001	>0.10	>0.02
	Cholesterol ester fatty acids					Cholesterol ester fatty acids				
Under 1 yr.										
Mean	125	33.4	0.8	19.0	0.2	67	7.7	6.7	1.7	0.02
SEm	5.2	1.4	0.3	1.6	0.1	3.9	0.4	0.4	0.3	0.01
Over 1 yr.										
Mean	159	38.4	0.5	15.3	0.1	105	8.1	7.6	1.8	0.0
SEm	11.4	1.2	0.2	0.9	0.1	9.1	0.3	0.7	0.5	0.0
P	<0.01	<0.001	>0.10	<0.01	>0.50	<0.001	>0.10	>0.10	>0.50	>0.05
	Glyceride fatty acids					Glyceride fatty acids				
Under 1 yr.										
Mean	64	15.8	1.9	4.9	0.1	65	9.6	1.9	0.1	0.0
SEm	5.7	0.7	0.3	0.7	0.1	5.6	0.8	0.3	0.08	0.0
Over 1 yr.										
Mean	45	15.3	1.2	3.9	0.2	54	9.1	2.6	0.4	0.2
SEm	7.3	2.1	0.3	0.6	0.1	5.0	1.3	0.6	0.2	0.1
P	<0.001	>0.50	>0.10	>0.01	>0.50	<0.001	>0.50	>0.10	>0.10	>0.05

[1] Control animals, 5 under one year and 5 over one year; fat-deficient animals, 6 over one year and 6 under one year.
[2] As percent of fatty acids in each fraction.
[3] "P" was derived from calculated "t" values (when $N < 30$) using a two tail test of the one tail probability table in Dixon and Massey, '51.

Influence of age on distribution of fatty acids

A statistical summary of the data for control and fat-deficient animals with respect to age is given in table 2. During the experimental period when blood samples were drawn the age of dogs under one year ranged from three to 12 months. The age of dogs over one year ranged from 17 to 42 months. In the control group, the most outstanding effect of age was a significantly lower level for dienoic acid in the total, phospholipid and cholesterol ester fractions for animals under one year of age. In the fat-deficient group, both the di- and trienoic acid levels in the total and phospholipid fractions were significantly lower in the younger dogs. There were no real differences in the distribution of the 2, 3, 4 or 6 double-bond fatty acids between the young and older dogs in either group but the amount of glyceride fatty acids was significantly higher in the dogs under one year of age.

DISCUSSION

Although phospholipids in blood serum are often considered to be the important carriers of unsaturated fatty acids, the data presented show that in healthy dogs, cholesterol esters in serum are equally important in this respect. The highest percentage (53 to 57%) of the dienoic (linoleic) acid present in serum of healthy dogs was bound as cholesterol esters. Kelsey and Longenecker ('41) have reported similar findings for beef plasma. In addition, the cholesterol ester fraction of control dog serum contained an appreciable amount of tetraenoic (arachidonic) acid representing about 25% of the total present in the serum. The highest percentage (60 to 70%) of the tetraenoic acid and about 37% of the dienoic acid in serum was present in the phospholipid fraction. However, when dogs were deprived of a dietary source of linoleic acid, they had very little linoleic and arachidonic acid in their blood serum. The high percentage of trienoic acid which they synthesized occurred mostly in the phospholipid fraction (60 to 70%). If fat-deficient animals make

this trienoic acid in an attempt to meet their metabolic needs for an nnsaturated fatty acid, it would appear that their requirement for an essential fatty acid is for an active component of phospholipids. Metabolically, however, this trienoic acid may act as an inhibitor for the normal maturation of cells, particularly of the epidermis as evidenced by the marked histological changes in the epidermal cells of fat-deficient dogs (Hansen and co-workers '51, '54).

When the diet of the dog provides sufficient linoleic acid to prevent the development of fat deficiency symptoms, he synthesizes arachidonic acid to such a degree that the blood serum has approximately equal amounts of di- and tetraenoic acid. This level of tetraenoic acid is considerably higher than that which has been found in the serum of healthy human subjects (Wiese, Gibbs and Hansen, '54; Evans et al., '56). On the other hand, the dienoic acid level in human subjects greatly exceeds that of the tetraenoic acid. Serum levels for di-, tri, and tetraenoic acids of healthy and fat-deficient rats are similar to those for the dog.[2] These species differences suggest two questions: (1) do the requirements for essential fatty acids differ in various species (linoleic for one, arachidonic for another); (2) is arachidonic acid the predominant unsaturated fatty acid in serum phospholipids of healthy human subjects?

With increasing interest in the essentiality of unsaturated fatty acids in nutrition, it is becoming more important to identify these fatty acids in biological materials. Mead and co-workers have done this for the rat (Mead, Steinberg and Howton, '53; Mead and Slaton, '56; Steinberg, Slaton, Howton and Mead, '56). By the use of C^{14}-labeled carboxyl carbon they have confirmed the work of Bernhard and Schoenheimer ('40) which showed that linoleic acid is not synthesized by the rat, hence is an essential dietary fatty acid. In addition, Mead and co-workers have demonstrated that linoleic acid is a precursor of arachidonic acid and the trienoic acid which is made by fat-deficient rats was found

[2] Panos and Wiese, unpublished data.

to be 5, 8, 11-eicosatrienoic acid and not linolenic acid. The latter finding would suggest the need for further identification of the penta- and hexaenoic acids which are found in blood and tissues if an exact evaluation of their importance in metabolic processes is to be made. It would appear from the data now available with respect to their small concentration in blood serum in relation to the intake of fat that these fatty acids are relatively less important than are the di-, tri- and tetraenoic acids in human nutrition. In the results reported for healthy and fat-deficient dogs, which represent two extremes insofar as the intake of fat and linoleic acid are concerned, significant differences in the amount of hexaenoic acid were observed only in the phospholipid fatty acid fraction. The total amount of this unsaturated fatty acid represented a very small fraction of the unsaturated fatty acids in the serum. From the absorption data for pentaenoic acid, one would conclude that the amount of this fatty acid also is of the same order of magnitude as hexaenoic acid. The large amounts of di- and tetraenoic acids found in the serum of healthy subjects would seem to indicate a dietary requirement for linoleic or arachidonic acid. The increased synthesis of a trienoic acid by infants and experimental animals when the diet is lacking in linoleic acid is more difficult to understand, particularly because increased amounts of this metabolite in the body do not prevent the development of skin changes which are so characteristic of a dietary deficiency of linoleic acid in young subjects.

SUMMARY

The distribution of the essential fatty acids in blood serum as influenced by diet has been determined in the phospholipid, cholesterol ester and glyceride fractions for 10 healthy control and 12 fat-deficient dogs. In the diet of healthy animals, dietary fat and linoleic acid constituted 30% and 4% of the calories, respectively. In the fat-deficient group, these were 1% and 0.1%, respectively. Spectrophotometric analysis was used for determination of the 2, 3, 4, 5 and 6 double-bond

fatty acids. In control animals, there were appreciable amounts of di- and tetraenoic acids present in the phospholipids and cholesterol esters with tetraenoic (arachidonic) acid predominating in the former and dienoic (linoleic) predominating in the latter fraction. There was very little tri-, penta- and hexaenoic acid in the serum of healthy dogs. In fat-deficient dogs, serum levels were low for di-, tetra- penta- and hexaenoic acids in all fractions, but were high for trienoic acid in the phospholipid and cholesterol ester fractions. Trienoic acid was the predominant unsaturated fatty acid in the phospholipids of these animals. Glycerides for both groups of dogs contained more dienoic than tri-, tetra-, penta- and hexaenoic acid.

Significant differences in fat levels and distribution of the unsaturated fatty acids between male and female dogs were not demonstrated. However, young control animals showed lower levels for dienoic acid than adult animals. Young animals in the low-fat group had lower levels for di- and trienoic acids than adult dogs.

LITERATURE CITED

BERNHARD, K., AND R. SCHOENHEIMER 1940 The inertia of highly unsaturated acids in the animal, investigated with deuterium. J. Biol. Chem., *133:* 707.

BLOOR, W. R. 1924 The fatty acids of blood plasma: II. The distribution of the unsaturated acids. Ibid., *59:* 543.

———— 1929 The oxidative determination of phospholipid (lecithin and cephalin) in blood and tissue. Ibid., *82:* 273.

DIXON, W. J., AND F. J. MASSEY 1951 Introduction to Statistical Analysis. McGraw Hill, New York.

EVANS, J. D., J. M. WALDRON, N. L. OLEKSYSHYN AND R. W. RIEMENSCHNEIDER 1956 Polyunsaturated fatty acids in normal human blood. J. Biol. Chem., *218:* 255.

HANSEN, A. E., AND H. F. WIESE 1951 Fat in the diet in relation to nutrition of the dog: I. Characteristic appearance and gross changes of animals fed diets with and without fat. Texas Repts. on Biol. and Med., *9:* 491.

HANSEN, A. E., S. G. HOLMES AND H. F. WIESE 1951 Fat in the diet in relation to nutrition of the dog: IV. Histologic features of skin from animals fed diets with and without fat. Ibid., *9:* 555.

HANSEN, A. E., J. G. SINCLAIR AND H. F. WIESE 1954 Sequence of histologic changes in skin of dogs in relation to dietary fat. J. Nutrition, *52:* 541.

HANSEN, A. E., H. F. WIESE, M. LAWLIS, D. J. D. ADAM AND M. A. BAUGHAN 1955 Effect of dietary fat on unsaturated fatty acids of serum of infants. Federation Proc., *14:* 436.

HANSEN, A. E., H. F. WIESE, D. J. D. ADAM, A. N. BOELSCHE AND M. E. HAGGARD 1957 The intake of linoleic acid in relation to serum unsaturated fatty acids and fat deficiency symptoms in infants. A. M. A. J. Dis. Child., *94:* 398.

KELSEY, F. E. 1939 The use of lipase in lipid analyses: I. Specificity of castor bean lipase. J. Biol. Chem., *130:* 187.

KELSEY, F. E., AND H. E. LONGENECKER 1941 Distribution and characterization of beef plasma fatty acids. Ibid., *139:* 727.

MEAD, J. F., G. STEINBERG AND D. R. HOWTON 1953 Metabolism of essential fatty acids: I. Incorporation of acetate into arachidonic acid. Ibid., *202:* 683.

MEAD, J. F., AND W. H. SLATON 1956 Metabolism of essential fatty acids: III. Isolation of 5, 8, 11-eicosatrienoic acid from fat-deficient rats. Ibid., *219:* 705.

STEINBERG, G., W. E. SLATON, D. R. HOWTON AND J. F. MEAD 1956 Metabolism of essential fatty acids: IV. Incorporation of linoleate into arachidonic acid. Ibid., *220:* 257.

WIESE, H. F., AND A. E. HANSEN 1951 Fat in diet in relation to nutrition of the dog: II. Lipid composition of tissues of animals fed diets with and without fat. Texas Repts. on Biol. and Med., *9:* 516.

III. Spectral analysis for unsaturated fatty acid content of tissues from animals fed diets with and without fat. Ibid., *9:* 545.

——— 1953 Semimicromethod for unsaturated fatty acids of blood serum. J. Biol. Chem., *202:* 417.

WIESE, H. F., R. H. GIBBS AND A. E. HANSEN 1954 Essential fatty acids and human nutrition: I. Serum level for unsaturated fatty acids in healthy children. J. Nutrition, *52:* 355.

WIESE, H. F., A. E. HANSEN, D. J. D. ADAM AND M. A. BAUGHAN 1956 Effect of intake of linoleic acid on unsaturated fatty acids of serum of infants. Feredation Proc., *15:* 577.

OXIDIZED PYRIDINE NUCLEOTIDES IN VARIOUS FRACTIONS OF THE BLOOD AND NIACIN AND TRYPTOPHAN METABOLITES IN THE URINE OF WOMEN ON A CONTROLLED ADEQUATE DIETARY [1]

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(Received for publication July 5, 1957)

No study has been found in the literature reporting daily values for niacin metabolism in both blood and urine of normal persons on a controlled adequate dietary of ordinary foods. From the work of Goldsmith and associates ('55) and Horwitt and coworkers ('56), the relationship of nicotinic acid and tryptophan to the prevention and cure of pellagra has been well established. It appears that an optimal intake of niacin, expressed as "niacin equivalents" to include the contribution of tryptophan, lies somewhat above the 8 to 10 mg a day necessary to prevent clinical signs of this nutritional deficiency. The supporting studies of urinary excretion of metabolites, particularly in association with "corn diets" (Goldsmith, '56; Frazier et al., '55) suggest that urinary N^1-methylnicotinamide (N^1MN) and its 6-pyridone can serve as indices of satisfactory niacin nutrition, but that in severe

[1] Technical Paper no. 1071, Oregon Agricultural Experiment Station.

[2] In this paper, the data on blood are taken from the thesis presented by Nina H. Morley to the Graduate School of Oregon State College in partial fulfillment of the requirements for the Ph.D. degree, June 1957; Present address: Dept. of Opthalmology, Banting Institute, Toronto, Canada.

deficiency the 6-pyridone may be present in concentrations too low for significant results (Horwitt et al., '56; Goldsmith et al., '52).

Analysis of a particular fraction of the blood, which has proved a convenient and valuable approach to the study of certain other nutrients, has so far failed to demonstrate for niacin any specific relationship of concentration of metabolites with changes in dietary intake or appearance of clinical symptoms. In the recent report of Vivian and others ('57) however, correlated blood and urine data indicated that the pyridine nucleotide (PN) content of the whole blood varied for subjects on a low niacin intake according to the amount of tryptophan available for niacin synthesis. Moreover, Horwitt and associates ('56) point out that even with decided changes in dietary, long term studies are desirable for reliable evaluation of results. Thus there is an apparent need for further data, collected under well-defined and controlled conditions, before parameters for optimal niacin status can be established for both blood and urine.

The purpose of the present study was to obtain daily fasting values for niacin metabolites in whole blood and its fractions and values for concurrent 24-hour urinary excretion of niacin and tryptophan products for 4 women receiving a constant adequate controlled diet of ordinary foods for a 30-day period.

EXPERIMENTAL

Diet

The controlled diet, patterned after that of Gifft and Hauck ('46) and used in this laboratory for previous investigations (Louhi et al., '52), provided daily, according to calculation, approximately 2000 calories,[3] 60 gm of protein,[3] 770 mg of tryptophan [4] and 8.7 mg of niacin [5] estimated microbiolog-

[3] Agricultural Handbook No. 8 ('50).

[4] Tryptophan values were calculated from data obtained by personal communication from Bernice K. Watt, Nutrition Analyst, U. S. Dept. of Agriculture, 1957.

[5] Analysis of niacin in foods was made by Frances Duryee using *Lactobacillus plantarum*.

ically. With the daily supplement of 0.3 and 0.5 mg of crystalline thiamine and riboflavin, respectively, in 0.1 *N* HCl, the 1953 recommended allowances of the National Research Council were met for all other known nutrients. This intake was kept constant throughout the 30-day period.

Subjects

The subjects selected were 4 women, two graduate students and two staff members, aged 23 to 49 years. All appeared healthy and were active in the experimental work. Three subjects remained at the same weights of 55, 62 and 70 kg but the 4th, FD, who began considerably above her ideal weight at 110 kg lost about 3 kg during the controlled period.

Preparation and analysis of blood samples

About 3 ml of blood were collected daily before breakfast by finger puncture to serve for the hematocrit and the 11 other determinations in triplicate being made in the laboratory at this time on each of these subjects. Trichloroacetic acid filtrates were prepared immediately for whole blood and its fractions and were analyzed at once in the case of the white cells. For the other fractions the filtrates were frozen and analyzed later. Control filtrates were prepared for each fraction from a venous sample of blood and a sufficient number of aliquots were stored frozen to allow parallel analyses with the filtrate samples for the day.

Direct analyses of the oxidized pyridine nucleotides in whole blood, red and white blood cells were made by the method of Burch and associates ('55). Since these workers had shown enzymatically that most of the PN in these blood fractions was present as diphosphopyridine nucleotide (DPN) the results of the present study have been expressed as DPN. Standard solutions were prepared from DPN [6] assayed by the methods

[6] Diphosphopyridine nucleotide was purchased from Sigma Chemical Co., St. Louis, Mo.

of Colowick et al. ('51) and LePage ('47) to be 78% pure. The weight of the white cells corresponding to each aliquot of filtrate was measured indirectly by analysis of the cell residues for acid-insoluble phosphate (Bessey et al., '47). In the internal standard method of analysis of serum, N^1methylnicotinamide,[7] added as a standard solution, was recovered from the serum filtrate. The serum blank was made by reversing the order of addition of NaOH and HCL solutions (Carpenter and Kodicek, '50). For each entire blood fraction series, both the 1 *N* NaOH reagent, prepared in one lot from a saturated solution and stored tightly sealed in individual tubes, and the 0.12 *N* HCl reagent were standardized for normality to ensure a pH of 1.8 to 1.9 in the final reaction mixture. Measurement of fluorescence was made in a Farrand fluorometer.

Preservation and analysis of urine samples

The 24-hour urine samples were preserved under toluene in amber bottles in the refrigerator. At two day intervals composites for each subject were made and stored frozen in several small bottles for the later analyses of N^1MN and its 6-pyridone fluorometrically (Huff and Perlzweig, '47; Rosen et al., '49) and for niacin, quinolinic acid and tryptophan microbiologically. In the microbiological assays, measurement was made in a Klett colorimeter of the turbidity produced by *Lactobacillus plantarum (L. arabinosus)* in diluted neutralized urine added to the appropriate Difco medium[8] (A.O.A.C., '55; Greene and Black, '44). Niacin was assayed as the free form. Quinolinic acid was estimated indirectly, by means of a conversion factor derived daily, from the increase in apparent niacin content of the urine following hydrolysis (Sarett, '51). Tryptophan was determined before and after hydrolysis with NaOH solution in the presence of cysteine and with reference to the daily standard curve for L-tryptophan. The values

[7] N^1-methylnicotinamide was purchased from Hoffman-LaRoche Inc., Nutley, N. J.
[8] Difco Laboratories Inc., Detroit, Mich.

reported for total tryptophan are derived from the formula: [9] total tryptophan = 2 (hydrolyzed tryptophan − free tryptophan) + free tryptophan. Analyses were made also for total nitrogen by a macro-Kjeldahl method and for creatinine [10] by the Folin method (Hawk et al., '47).

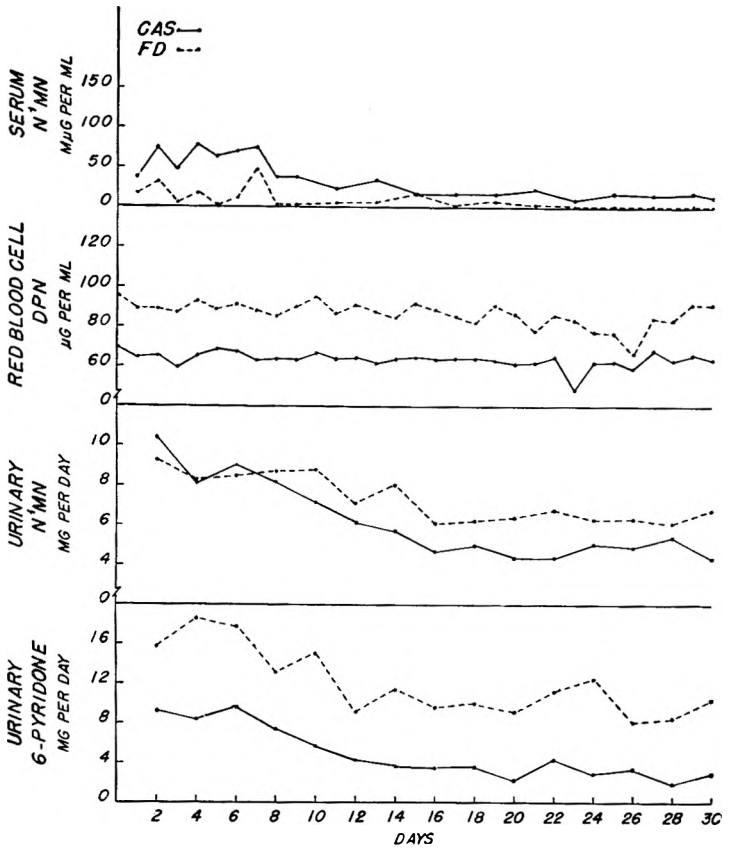

Fig. 1 Blood and urine values for two subjects on a controlled diet

[9] This formula for calculating total tryptophan was received in personal communication with Alice B. Stanfield, University of Arizona, Tucson, Arizona and is supported by observations in our laboratory and by the work of Krehl et al. ('46) that L-tryptophan is not racemized during the hydrolysis of urine.

[10] Analysis of urinary creatinine was made by Muriel Woodring.

RESULTS

Blood analyses

The levels of DPN obtained for red blood cells were maintained quite uniformly throughout the controlled diet period within narrow ranges characteristic for each subject. This is illustrated in figure 1 for CAS and FD. The curves of the other subjects are parallel and intermediate. Similar patterns were obtained for whole blood DPN but the irregularity observed with the white cells was doubtless attributable in large part to the extra analytical procedure required for this fraction. Control filtrates gave concurrent results and the coefficients of variation of 3 and 4% for whole blood and red cells demonstrated the reliability of the method and the stability of DPN in frozen filtrates. The fact that the values in the white cell and serum series during the first week were low for the control as well as for the test filtrates suggested that the data for this period be omitted from calculations for these fractions.

The group means of 34.0 and 75.3 μg of DPN/ml of whole blood and red blood cells respectively, and 87.9 μg/gm of white blood cells, as well as the ranges of the subjects for these fractions, agree with the corresponding sets of values reported for normal persons by other laboratories using similar fluorometric methods (table 1). It can not be assumed, however, that this agreement implies adequacy of niacin status since pellagrins have been observed to have red cell DPN values in the same range as normal persons, although generally toward the lower end (Mezincescu and Dumitrescu-Opreanu, '54; Kohn et al., '39). These investigators noted that the group mean and spread bear greater clinical significance than the individual values. In the present study the high group means and narrow spread reflect favorably upon the adequacy of niacin metabolism of these subjects.

In the serum filtrate analyses, the trend, over and above the daily fluctuations of the first days, for three of the women toward attaining a similar level of N^1MN and holding this

TABLE 1

Niacin metabolites in blood and niacin and tryptophan products in urine of normal subjects

MEASUREMENT	LABORATORY			
Blood analyses	This study	Burch et al. ('55)	Kerppola and Pätiälä ('54)	Levitas et al. ('47)
No. of subjects	4 (30 days)[1]	8-12	40	10-16
Whole blood DPN,	34.0[2]	29.8	28.1	36
μg per ml	30.0-37.1[3]	23.6-36.2	20.0-37.1	28-44
Rbc DPN,	75.3	68.0	67.1[4]	77
μg per ml	63.4-86.8	58.0-86.0	51.7-90.0[4]	62-89
Wbc DPN,	87.9	90.0		
μg per gm	82.5-94.4	76.0-101.0		
Serum N^1MN,	0.017	0.46		0.14[5]
μg per ml	0.003-0.023	0.36-0.52		0.04-0.24[5]
Urine analyses	This study	Frazier et al. ('55)[6]	Sarett ('51)[7]	
No. of subjects	4 (30 days)	6	3	
N^1MN,	5.8	6.1	3.0	
mg per day	4.5-7.3	6.3-7.6	2.3-3.4	
6-Pyridone,	7.3	7.1	3.6	
mg per day	4.9-12.0	5.1-9.8	3.2-4.0	
Nicotinic acid,	0.44	0.52	0.56	
mg per day	0.36-0.54	0.49-0.55	0.51-0.58	
Free tryptophan,	12.9		12.6	
mg per day	10.9-15.1		11.7-14.9	
Total tryptophan,	25.4[8]			
mg per day	21.3-32.0[8]			
Quinolinic acid,	4.9	3.3	4.8	
mg per day	3.7-6.4	2.8-3.6	4.1-6.5	

[1] For white blood cell and serum determinations, the last 24 and 23 days of the study.

[2] The single value represents the group mean in each instance.

[3] Range of mean values for subjects.

[4] Red blood cell DPN calculated by these authors from whole blood analysis and hematocrit determination.

[5] These values were calculated from the authors' data for DPN content of plasma.

[6] Values taken for control periods.

[7] These values were calculated from the author's data for unsupplemented periods.

[8] Total tryptophan = 2(hydrolyzed tryptophan − free tryptophan) + free tryptophan. See text.

level uniformly from the 16th day onward, may represent adaptation to a new dietary intake of niacin and tryptophan. A similar period of adjustment for subject FD resulted however in markedly low serum N^1MN values during the latter half of the study. The means of 0.063 and 0.019 μg of N^1MN/ml of serum for days one to 8 and the corresponding 0.023 and 0.003 for the last 23 days based on the analysis of two-day pooled filtrates suggest that the intake of the above nutrients had been higher for subjects CAS and FD previous to the study (fig. 1).

These ranges and the group mean of 0.017 μg/ml for the last 23 days, were considerably lower than any values in the literature for the niacin equivalent content of serum or plasma. It is not clear if the subjects from the other laboratories referred to in table 1 were receiving amounts of niacin comparable to those in this study or if the blood had been collected under fasting conditions. According to the work of Burch and associates ('55) on rats, it would seem important to define dietary states in conjunction with serum N^1MN concentrations. No doubt some of the differences in results were due to difference in analytical procedure, including the selection of the filtrate blank system. It is felt that the method involving only the reversal of the acid and base addition should provide for closer duplication of final volume and ionic strength in test and blank solutions than other commonly practiced methods (Carpenter and Kodicek, '50).

Since the low content of N^1MN in the serum did not permit further dilution of the interfering substances which were found to enhance the fluorescence of the derivative of added N^1MN by 10 to 20% and to result in blank values of about 80% of the reading for the sample, the present analytical method is not considered sufficiently sensitive to derive valid quantitative information for serum.

The total DPN concentration of the whole blood, calculated from the contributions of each fraction, came within 10% agreement of the direct analysis for each subject. The factor for converting N^1MN content to the DPN equivalent, the ratio

of the fluorescence of the derivatives of DPN and N^1MN obtained in recovery from filtrate, was found to be 0.24. This agrees with other work (Duncan and Sarett, '51; Kirk, '54) but is much lower than the factor of 0.4 obtained by Levitas et al., ('47) and assumed by Kerppola and Pätiälä ('54).

Urine analyses

The values obtained for group means and ranges for the urinary excretion of N^1MN, 6-pyridone, nicotinic acid, tryptophan and quinolinic acid were similar to those derived from data for unsupplemented periods in two other laboratories where these metabolites were determined in a comparable manner. Inasmuch as the levels for urinary N^1MN and 6-pyridone found with the present dietary intake were higher than those for the three subjects of Sarett ('51), who received about the same amount of niacin but 15 gm less protein per day, and were not lower than those for the subjects of Frazier and coworkers ('55) on their control diet providing 2.6 mg of niacin and 115 mg of tryptophan more per day, it can be assumed that the 4 women on the 30-day study received adequate amounts of niacin and its precursor. Neither quinolinic acid nor tryptophan excretion have been found to vary to any marked extent with diet (Horwitt et al., '56). Any differences in the ranges reported here are probably due to assay techniques rather than to amino acid intake (Eckhardt and Davidson, '49).

Figure 1 illustrates for urinary excretion as it does for blood levels, the regulated but individual nature of niacin and tryptophan metabolism. In the decreasing excretion of N^1MN and 6-pyridone during the first 14 days, subjects CAS and FD exhibit parallel tendencies, resembling the trends in serum N^1MN, to adapt to a new dietary intake. Aside from the observation that FD held the highest position in the group for all the analyses except for serum N^1MN, urinary niacin and tryptophan, there appeared to be no correlation between the concentration of any of the metabolites studied and weight, size, age, creatinine coefficient or urine volume.

DISCUSSION

With a constant adequate dietary intake, especially of natural rather than synthetic foods, one would expect to obtain stable values in blood and urine analyses which would represent the levels of normal individuals with tissues well-

TABLE 2

Mean values for urinary excretion of nitrogen, niacin and the ratio of 6-pyridone to N¹MN for three periods of controlled dietary

URINARY MEASUREMENT	DAYS	CAS	FD	NM	AS
Total nitrogen,[1] gm per day	1–10	10.7	9.4[2]	8.7	10.2
	11–20	9.1	9.7	8.4	9.0
	21–30	8.9	9.2	8.1	9.2
Niacin equivalents,[3] mg per day	1–10	14.8	21.2	9.6	9.0
	11–20	8.0	14.5	9.3[4]	10.4
	21–30	7.4	14.3	8.3	10.6
Ratio, 6-pyridone to N¹MN[5]	1–10	0.83	1.64	1.09	1.56
	11–20	0.60	1.31	0.87	1.23
	21–30	0.58	1.39	0.79	1.49

[1] If an output of 1 gm fecal nitrogen is assumed, 8.9 gm urinary nitrogen per day would represent a balance for the constant calculated intake of 9.9 gm nitrogen.

[2] This value is the mean of 15.0, 6.0, 5.4, 10.6 and 9.9.

[3] Urinary niacin equivalents signifies the total daily urinary excretion of N¹MN, 6-pyridone and niacin calculated as niacin equivalents according to molecular weight. The excretion would balance the intake at 8.7 mg.

[4] This value is the mean of 11.9, 9.5, 8.9, 8.6 and 7.7 mg per day niacin equivalents.

[5] Both N¹MN and 6-pyridone were calculated as niacin equivalents.

supplied in respect to niacin. It is noteworthy that in spite of the uniformity of intake the metabolic pattern was distinctive for the 4 women studied. Any tendency for change was in the direction of narrowing the spread of values for the group. According to the suggestion of Goldsmith ('56), a satisfactory supply of niacin in the tissues was indicated for each subject by the excretion of more than 0.6 mg per day of N¹MN plus 6-pyridone for each milligram of niacin ingested.

In fact, for the first two weeks of the study, all 4 subjects excreted more niacin equivalents of N^1MN, 6-pyridone and niacin per day than the dietary intake of 8.7 mg niacin. This extra niacin was presumably contributed by body stores and from conversion of tryptophan. Since FD and AS continued in this excess of excretion over intake throughout the experimental period (table 2), it might be suggested that these two subjects had either a lower metabolic need for niacin or a greater capacity for tryptophan conversion than did CAS or NM. Some confirmation of this higher conversion rate is shown in the accompanying excretion of about 1%, as against 0.6% for CAS and NM, of the tryptophan ingested, as quinolinic acid (expressed as tryptophan equivalents). Frazier et al. ('55) had observed slightly increased outputs of quinolinic acid following a dose of 100 mg of tryptophan.

It would seem likely that the inadequate supply of calories to FD, as evidenced by the loss of weight during the study, would increase the demand on dietary protein for calorie needs and consequently reduce the tryptophan available for conversion to niacin. On the other hand, catabolized body tissue might actually provide extra tryptophan (Horwitt, '55) as well as release stores of niacin (Bessey et al., '56). Although no "flooding" of niacin was observed in the serum, the levels of urinary tryptophan and niacin products, as well as a continued tendency to a negative nitrogen balance, appear to be the effect of tissue catabolism. Since it is probable, although not determined by analysis, that the fecal nitrogen amounted to approximately 1 gm per day, it appears that subject AS, with excretions of over 9.0 gm urinary nitrogen per day, was also in slight negative nitrogen balance for the whole period of investigation. There was no demonstrable loss in weight for AS but, as shown in table 2, her pattern of urinary excretion somewhat resembled that of FD.

These two subjects also differ from CAS and NM in excreting a higher proportion of 6-pyridone to N^1MN, as calculated in niacin equivalents (table 2). The difference in these ratios, which did not arise from a difference in dietary intake, empha-

sized the fact that individual variability, also observed by Frazier et al. ('55), would render this ratio of questionable value in measuring adequacy of niacin status. Indeed, the ratios obtained for our subjects were in the reverse order to expectations based on correlation with a niacin intake-excretion balance as suggested by Frazier and associates ('55). Since it has been noted (Sarett, '51) that the 6-pyridone to N^1MN ratio was higher after supplementation with tryptophan than with quinolinic acid, one might conjecture that the difference shown in this ratio by subjects AS and FD represented the use of a greater amount of tryptophan for niacin synthesis than in the other two women. Further investigation of this ratio under varying conditions might prove fruitful.

A study of normal individuals under controlled adequate dietary conditions offers a measure of the variability to be expected in the daily analyses of blood and urine when there is no influence from a change in diet. Of the blood fractions, the red blood cell and whole blood gave the most uniform results, with coefficients of variation of about 7% for each subject over the whole period. According to the analyses of the control filtrate samples, 3 and 4% for each of the above fractions, respectively, represented daily variability of the method. Excellent consistency was evidenced in the results of the control samples of urine for all the products. The coefficients of variation of 15 to 50 and 16 to 30% for the daily content of 6-pyridone and N^1MN in the test urines, in contrast to approximately 10% for the corresponding variation in the other substances measured, can be attributed to the immediate response in the output of these two particular metabolites to change in diet. These results emphasize the need for careful attention to the length of an adjustment period in evaluating excretion levels of 6-pyridone and N^1MN. This seems particularly necessary since the interrelationship of tryptophan and niacin involves the consideration of the protein and calorie needs of the body. Furthermore Horwitt et al., ('56) have remarked on the time required to achieve a steady state for nitrogen excretion in their subjects.

The red blood cell and whole blood concentrations no doubt reflect the store of pyridine nucleotides in the body tissues as a whole, and no immediate change in content would be expected for normal persons on a constant adequate diet. It seems unlikely that synthesis of DPN can occur in the mature human erythrocyte (Hogeboom and Kuff, '55; Malkin and Denstedt, '56; Preiss and Handler, '57). It is probable therefore that only slight gradual changes in the absolute content of red cell PN could be detected under any experimental condition. It is stimulating to think that any variation in red cell PN may be of kind and state rather than of marked change in amount (Reinhold and coworkers, '53).

SUMMARY

Daily fasting levels of oxidized pyridine nucleotides (expressed as DPN) in whole blood and blood cells and of N^1-methylnicotinamide (N^1MN) in serum were determined fluorometrically for 4 women receiving for 30 days a controlled dietary containing 8.7 mg of niacin and 770 mg of tryptophan. Concomitant 24-hour urinary excretions were analysed for N^1MN, 6-pyridone, niacin, tryptophan and quinolinic acid. Determinations of hematocrit value and urinary nitrogen and creatinine provided additional information.

The group means and ranges for the DPN content of whole blood and red blood cells for the whole period were 34.0 (30.0 to 37.1) and 75.3 (63.4 to 86.8) ug/ml respectively. For the last 23 days of the study, the corresponding values for DPN in white blood cells were 87.9 (82.5 to 94.4) μg/gm and for N^1MN in serum, 0.017 (0.003 to 0.023) ug/ml.

There was little variation in the day to day content of DPN in the red blood cells or whole blood for any of the subjects as evidenced by a mean of 7% for the coefficients of variation for the 30 days. Coefficients of variation of 3 and 4% respectively for the results of the analyses of the control filtrates of these two blood fractions indicated that these were the most reliable fractions to measure. The white blood cell and serum

levels showed a greater variation but maintained the trend of individual patterns of concentration found in all fractions.

The means and ranges of the urinary niacin, N^1MN and 6-pyridone were 0.44 (0.36 to 0.54), 5.8 (4.5 to 7.3) and 7.3 (4.9 to 12.0) mg/day respectively. Corresponding values for free and total tryptophan and for quinolinic acid were 12.9] (10.9 to 15.1), 25.4 (21.3 to 32.0) and 4.9 (3.7 to 6.4) mg/day.

For all subjects a period of adjustment of up to 16 days was indicated by the change in levels of serum N^1MN, and urinary N^1MN and 6-pyridone.

Two subjects were alike in a tendency to negative nitrogen balance, negative niacin balance and a ratio of 6-pyridone to N^1MN exceeding one.

LITERATURE CITED

AGRICULTURAL HANDBOOK No. 8 1950 Composition of Foods: Raw, Processed, Prepared. U. S. Dept. of Agriculture.

A. O. A. C. 1955 Official Methods of Analysis. Washington, D. C.

BESSEY, O. A., M. K. HORWITT AND R. H. LOVE 1956 Dietary deprivation of riboflavin and blood riboflavin levels in man. J. Nutrition, *58:* 367–383.

BESSEY, O. A., O. H. LOWRY AND M. J. BROCK 1947 The quantitative determination of ascorbic acid in small amounts of white blood cells and platelets. J. Biol. Chem., *168:* 197–205.

BURCH, H. B., C. A. STORVICK, R. L. BICKNELL, H. C. KUNG, L. G. ALEJO, W. A. EVERHART, O. H. LOWRY AND O. A. BESSEY 1955 Metabolic studies of precursors of pyridine nucleotides. Ibid., *212:* 897–907.

CARPENTER, K. J., AND E. KODICEK 1950 The fluorometric estimation of N^1-methylnicotinamide and its differentiation from coenzyme I. Biochem. J., *46:* 421–426.

COLOWICK, S. P., N. O. KAPLAN AND M. M. CIOTTI 1951 The reaction of pyridine nucleotide with cyanide and its analytical use. J. Biol. Chem., *191:* 447–459.

DUNCAN, M., AND H. P. SARETT 1951 Effect of nicotinic acid and tryptophan on pyridine nucleotides of red blood cells in man. Ibid., *193:* 317–324.

ECKHARDT, R. D., AND C. S. DAVIDSON 1949 Urinary excretion of amino acids by a normal adult receiving diets of varied protein content. Ibid., *177:* 687–695.

FRAZIER, E. I., M. E. PRATHER AND E. HOENE 1955 Nicotinic acid metabolism in humans. I. The urinary excretion of nicotinic acid and its metabolic derivatives on four levels of dietary intake. J. Nutrition, *56:* 501–516.

GIFFT, H. H., AND H. M. HAUCK 1946 A comparison of four methods for studying the urinary excretion of thiamine. Ibid., *31:* 635–645.

GOLDSMITH, G. A. 1956 Experimental niacin deficiency. J. Am. Dietet. Assoc., *32:* 312–316.

GOLDSMITH, G. A., H. L. ROSENTHAL, J. GIBBENS AND W. G. UNGLAUB 1955 Studies of niacin requirement in man. II. Requirement on wheat and corn diets low in tryptophan. J. Nutrition, *56:* 371–386.

GOLDSMITH, G. A., H. P. SARETT, U. D. REGISTER AND J. GIBBENS 1952 Studies of niacin requirement in man. I. Experimental pellagra in subjects on corn diets low in niacin and tryptophan. J. Clin. Invest., *31:* 533–542.

GREENE, R. D., AND A. BLACK 1944 The microbiological assay of tryptophane in proteins and foods. J. Biol. Chem., *155:* 1–8.

HAWK, P. B., B. L. OSER AND W. H. SUMMERSON 1947 Practical Physiological Chemistry. 12th Ed., Blakiston, Philadelphia, Pa.

HOGEBOOM, G. H., AND E. L. KUFF 1955 Relation between cell structure and cell chemistry. Federation. Proc., *14:* 633–638.

HORWITT, M. K. 1955 Implications of observations made during experimental deficiencies in man. Ann. N. Y. Acad. Sciences, *63:* 165–174.

HORWITT, M. K., C. C. HARVEY, W. S. ROTHWELL, J. L. CUTLER AND D. HAFFRON 1956 Tryptophan-niacin relationships in man. J. Nutrition, *60:* sup. 1, 1–43.

HUFF, J. W., AND W. A. PERLZWEIG 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. II. A sensitive method for the determination of N^1-methylnicotinamide in urine. J. Biol. Chem., *167:* 157–167.

KERPPOLA, W., AND J. PÄTIÄLÄ 1954 Pyridinenucleotides (coenzyme I and II) of blood in normal subjects and in various diseases, especially tuberculosis. Ann. Med. Exper. et Biol. Fenniae (Helsinki), *32:* sup. 7, 3–30.

KIRK, J. E. 1954 Blood and urine vitamin levels in the aged. Symposium on Problems of Gerontology, Nutrition Series, *9:* 73–94. National Vitamin Foundation, Inc., N. Y.

KOHN, H. I., AND F. BERNHEIM 1939 The blood V-factor (coenzyme) level in normal and pathological subjects. J. Clin. Invest., *18:* 585–591.

KREHL, W. A., J. DE LA HUERGA AND C. A. ELVEHJEM 1946 Tryptophane studies. I. The effect of niacin on the utilization of tryptophane. J. Biol. Chem., *164:* 551–561.

LEPAGE, G. A. 1947 Preparation of diphosphopyridine nucleotide. J. Biol. Chem., *168:* 623–628.

LEVITAS, N., J. ROBINSON, F. ROSEN, J. W. HUFF AND W. A. PERLZWEIG 1947 The fluorescent condensation product of N^1-methylnicotinamide and acetone. III. A rapid fluorometric method for the determination of the total pyridine nucleotides in the red blood cells. Ibid., *167:* 169–175.

LOUHI, H. A., H. H. YÜ, B. E. HAWTHORNE AND C. A. STORVICK 1952 Thiamine metabolism of women on controlled diets. I. Daily urinary thiamine excretion and its relation to creatinine excretion. J. Nutrition, *48:* 297–306.

MALKIN, A., AND O. F. DENSTEDT 1956 The metabolism of the erythrocyte. XI. Synthesis of diphosphopyridine nucleotide in the erythrocyte. Can. J. Biochem. Physiol., *34:* 130–140.

MEZINCESCU, M. D., AND Z. DUMITRESCU-OPREANU 1954 Blood nicotinic acid in pellagra. Comun. Acad. Rep. Populare Române (Bucharest), *4:* 65–68. (microfilm)

PREISS, J., AND P. HANDLER 1957 Enzymatic synthesis of nicotinamide mononucleotide. J. Biol. Chem., *225:* 759–770.

REINHOLD, J. G., A. F. HOGANS AND L. M. HERSHENSON 1953 Pyridine nucleotide partition in red blood cells. Federation. Proc., *12:* 256–257.

ROSEN, F., W. A. PERLZWEIG AND I. G. LEDER 1949 A fluorometric method for the determination of the 6-pyridone of N^1-methylnicotinamide in urine. J. Biol. Chem. *179:* 157–168.

SARETT, H. P. 1951 Quinolinic acid excretion and metabolism in man. Ibid., *193:* 627–634.

VIVIAN, V. M., M. M. CHALOUPKA AND M. S. REYNOLDS 1957 Some aspects of tryptophan metabolism on low niacin intake. Federation. Proc., *16:* 401.

EFFECTS OF PROTEIN PER CALORIE RATIO AND DIETARY LEVEL OF FAT ON CALORIE AND PROTEIN UTILIZATION [1]

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(Received for publication July 6, 1957)

INTRODUCTION

The role of fat and carbohydrate as factors affecting protein utilization and metabolism has been extensively investigated during the past 100 years. The literature on the subject, which has been comprehensively reviewed by Munro ('51), indicates that the effects which have been reported fall into different categories depending on the experimental procedure and the condition of the experimental animals. In the first place, nitrogen retention is influenced by the caloric intake of the animal. Rosenthal and Allison ('56) suggest that there may be an optimum caloric intake for each level of dietary protein. Experiments on moderately undernourished animals, on animals receiving adequate diets and on surfeit-fed animals suggest that, as a source of calories, fat and carbohydrate are equally effective in sparing protein in mature animals when the diet contains all three components (Deuel, '48, '55; Swanson, '51; Munro '51; Calloway and Spector, '55; Metta and Mitchell, '56).

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. We are indebted to Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, for supplies of some of the crystalline vitamins.

Secondly, evidence from experiments in which animals have been fed on carbohydrate-free or protein-free diets suggests that carbohydrate plays a role in protein metabolism which cannot be taken by fat (Deuel, '48; Munro, '51; Thomson and Munro, '55; Cohn and Joseph, '56). Thirdly, Swanson ('51) and Deuel ('55) have presented evidence for a specific nitrogen-sparing effect of fat under conditions of severe caloric restriction. In contrast, Calloway and Spector ('55), in shorter experiments under similar conditions, and Rosenthal ('52), in experiments on dogs, observed no specific beneficial effect of fat. Finally, there is no evidence, according to Munro ('51) to indicate that there are differences in protein utilization during growth due to the carbohydrate or fat content of the diet. This view is generally supported by the observations of Forbes, Swift and associates (Forbes et al., '46 c, d; French, Black and Swift, '48) and of Metta and Mitchell ('56). However, Schreiber and Elvehjem ('55) noted in ad libitum feeding experiments using young growing rats that a progressive increase in the level of dietary fat resulted in an apparent increase in the efficiency of protein utilization when the protein per Calorie ratio (Prot./Cal.) was kept constant.

The protein per Calorie ratio has been recognized as an important factor in poultry nutrition (Combs and Romoser, '55; Leong et al., '55; Matterson et al., '55; Donaldson et al., '55; Hill and Dansky, '50; Sunde, '56). Donaldson, Combs and Romoser ('56) cited evidence from the literature, and from their own experiments with chicks, to show that growth, calorie utilization, protein utilization and carcass composition were influenced by the protein per Calorie ratio of the diet.

The results of previous studies on the effect of the level of dietary fat, and the observations of Schreiber and Elvehjem ('55) on the importance of the protein per Calorie ratio in studies with the rat, made it desirable to examine in greater detail the effects of the protein per Calorie ratio

of the diet and of the level of dietary fat on the utilization of calories and protein in the rat.

EXPERIMENTAL PROCEDURE

Male weanling albino rats of the Sprague-Dawley strain were used, 6 rats per group. The sources of protein, carbohydrate and fat were casein, sucrose and oleomargarine, respectively. In experiment 1, all three diets contained 10% of protein and three different levels of fat (0, 10 and 30%). In experiment 2, there were 4 series of diets, each having a different Prot./Cal.[2] Within each series there were three groups which received 0, 10 or 30% of fat. All diets contained 4% of Salts 4 (Hegsted et al., '41), and the following amounts of water soluble vitamins (in milligrams per kilogram of diet): thiamine · HCl, 16; riboflavin, 12; pyridoxine, · HCl, 8; niacin, 100; Ca-pantothenate, 80; inositol, 400; biotin, 0.8; vitamin B_{12}, 0.08; and choline · HCl, 4000. The above amounts of water-soluble vitamins were considered to be adequate even when the dietary fat was increased. The amounts of fat soluble vitamins per rat per week were: vitamin A, 1600 I. U.; vitamin D_2, 140 I. U.; α-tocopherol, 9.6 mg and menadione, 1.2 mg. These were administered with three drops of cottonseed oil. This supplement was also considered to satisfy the essential fatty acid requirement.

All experiments were of three weeks' duration. The rats were fed ad libitum. Food consumption and the nitrogen content of urine and feces were determined for the last three days of each week.

The caloric content of each diet was calculated on the basis of the following values (Sherman, '41): sucrose, 3.96 Cal.; casein, 3.90 Cal., (4.55 Cal. × N%/100 × 6.25); fat, 9.45 Cal. The nitrogen content of each diet was estimated by the Kjeldahl procedure. All diets were prepared just before the experiment and kept in the refrigerator during the period of experiment. Room temperature was about

[2] In these experiments Prot./Cal. represents milligrams of protein per Calorie.

24°C and the relative humidity was about 34%. The carcass analyses were carried out at the end of the second and third weeks on rats fed the diet containing 18 mg Prot./Cal. After killing the rats, the carcasses were opened and were dried to constant weight at 105°C for about 48 hrs. Each carcass was finely divided and the entirety of the dry residue was used for fat extraction.

RESULTS

Experiment 1. In this experiment all three diets contained the same amount of protein (10%). The protein per calorie ratios were 26.0, 23.0 and 18.4 for the diets containing 0%, 10% and 30% fat respectively. The results of the experiment are shown in table 1.

The group receiving the 10% fat diet showed the greatest body gain during the three-week period (100.8 gm), but the gain of the group receiving the fat-free diet was almost the same (98.8 gm). The gain for the 30% fat group was somewhat less than that of either of the other groups.

Body gain per Calorie consumed followed the same order. The average values for body gain/Cal. of the groups receiving no fat and 10% fat were 0.0713 and 0.0717 gm, respectively, but that of the 30% fat group was 0.0617 gm.

Body gain per gram of nitrogen consumed (Gain/N) did not differ much among the three groups, except for the somewhat higher value for the 10% fat group during the second week. There was no difference in the percentage of nitrogen retained by the different groups.

Experiment 2. In this experiment the rate of gain increased as the amount of protein per Calorie was increased (fig. 1). The three-week gains were between 25 and 40 gm with about 17 mg of Prot./Cal.; these rose to 105 to 125 gm with about 60 mg of Prot./Cal. Throughout the whole range of Prot./Cal. the gains for the 30% fat group were greater than those for the groups receiving no fat or 10% of fat. With higher Prot./Cal., groups receiving 10% of fat gained more rapidly than those receiving no fat.

TABLE 1

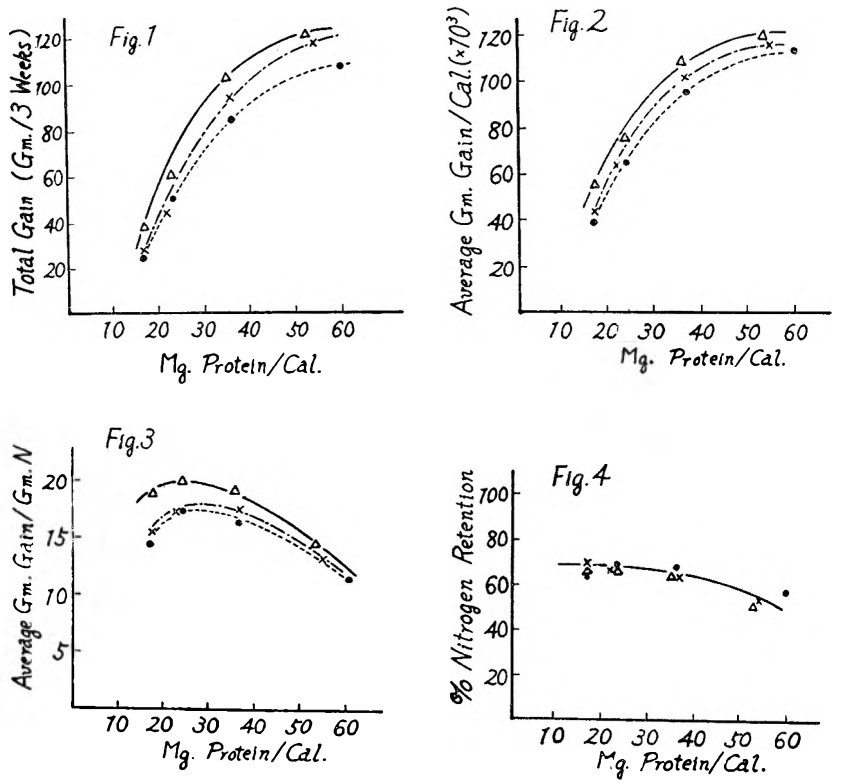
Effect of fat on protein and calorie utilization of rats fed diets containing 10% of casein [1]

FAT % IN DIET	1ST WEEK			2ND WEEK			3RD WEEK		
	0	10	30	0	10	30	0	10	30
Weight gain/week, *gm*	13.3	12.3	9.0	23.0	25.8	17.5	17.3	16.6	13.0
Food intake/day, *gm*	6.7	6.5	4.3	10.3	8.6	6.9	11.2	10.2	7.2
Cal. intake/day	25.7	28.2	23.0	39.6	37.3	30.8	43.0	44.3	38.8
N intake/day, *mg*	112	105	74	171	133	117	186	164	123
N Balance, *mg*	79.8	77.7	52.6	118.6	86.2	84.0	111.3	96.8	74.3
(Gain/Cal.) $\times 10^3$	74.0	62.4	56.0	83.0	98.8	81.1	57.5	53.5	48.4
Gain/N[2]	17.0	16.7	17.4	19.1	27.8	21.3	13.7	14.4	15.0
N retention, %	74.8	78.0	75.1	73.4	69.1	75.6	64.0	62.5	64.0
Total Cal. Intake							756	770	648
Total N intake, *gm*							3.28	2.82	2.20
Initial body wt., *gm*	45.1	45.1	45.5						
Final body wt., *gm*	58.5	57.8	54.5	81.5	83.5	72.1	98.8	100.8	85.1

[1] All values are averages for groups of 6 rats.
[2] Gram gain per gram nitrogen intake.

The values for average Gain/Cal. showed a similar trend (fig. 2). With 17 mg of Prot./Cal., Gain/Cal. was only 0.040 to 0.055 gm, but this increased gradually with each increase in Prot./Cal. until the values were between 0.11–0.12 with 60 mg of Prot./Cal. The 30% fat groups also showed somewhat higher values.

The curve for Gain/N with increasing Prot./Cal. was quite different from those for growth and Gain/Cal. (fig. 3). In this case, the Gain/N was 15 to 19 gm with 17 mg of



···•··· fat free group; —.—×—.— 10% fat group; —Δ— 30% fat group.

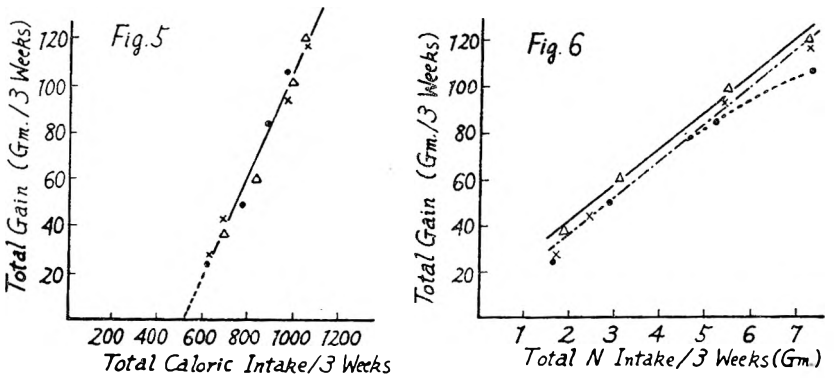
Effect of protein per Calorie ratio of the diet:

Fig. 1 On total gain for three weeks.
Fig. 2 On average gain per Caloric intake during three weeks.
Fig. 3 On average gain per nitrogen intake during three weeks.
Fig. 4 On average percentage nitrogen retention during three weeks.

Prot./Cal.; this increased to 17 to 20 gm with about 23 mg of Prot./Cal., and then decreased to 12 to 13 gm with about 60 mg of Prot./Cal. The 30% fat group showed higher values except at the 60 mg of Prot./Cal. level.

Nitrogen retention was between 65 and 70% in all cases except for the 60 mg of Prot./Cal. level in which case the values were lower (50 to 60%) (fig. 4).

The total gains for the three weeks were directly proportional to the total caloric intake during the period regardless of the fat or protein content of the diet (fig. 5). The exten-



··· ··· fat free group; — . — × — . — 10% fat group; — Δ — 30% fat group.

Fig. 5 Effect of total Caloric intake on total gain for three weeks.
Fig. 6 Effect of total nitrogen intake on total gain for three weeks.

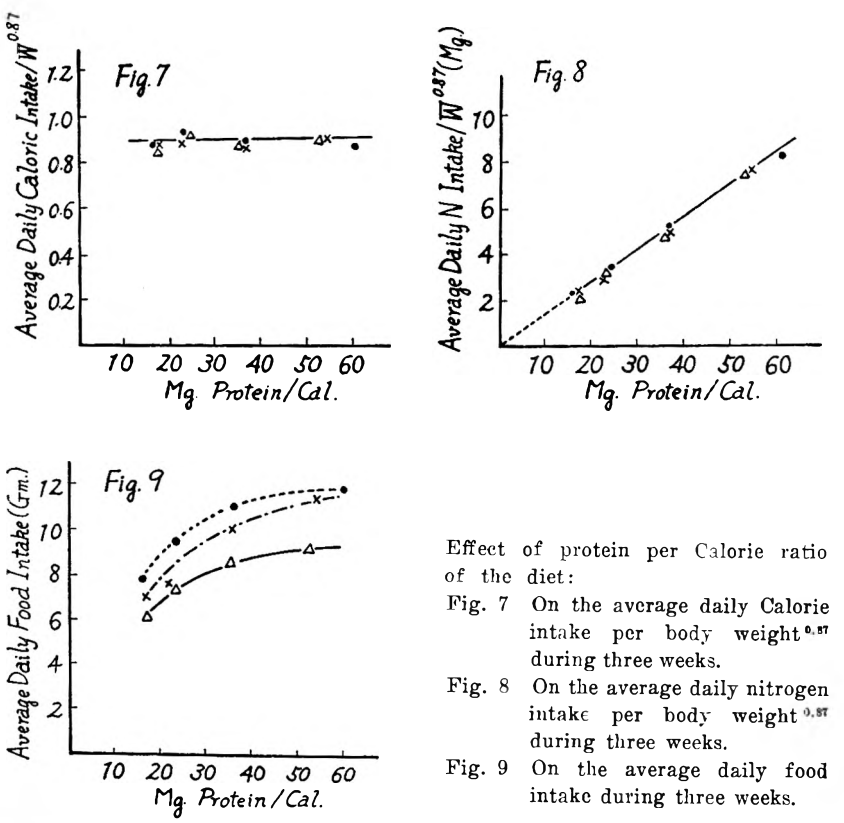
sion of the line crosses the abscissa at the point of 510 Cal. The slope of the line is 23 gm gain per 100 Cal. consumed.

The relationship between total gain and total nitrogen intake (fig. 6) was similar to that for total caloric intake (fig. 5) except that the 30% fat groups showed slightly higher values throughout whole range of Prot./Cal. in this experiment and the values for the 10% fat groups were somewhat greater at higher nitrogen intake than those of the groups receiving no fat.

Caloric intake per average weight to the 0.87 power (Caloric intake/$W^{0.87}$) (Hegsted and Haffenreffer '49) was

constant (fig. 7) regardless of the levels of fat and protein in the diets. Nitrogen intake per average weight to the 0.87 power (N intake/$W^{0.87}$) was proportional to the Prot./Cal. (fig. 8), and this line goes through the origin.

The average amounts of food consumed increased as the Prot./Cal. increased and at the same Prot./Cal., the amounts decreased when the fat level was raised (fig. 9). There were no differences in moisture content in the carcasses of rats kept on diets containing 17 mg of Prot./Cal. with various levels of fat for two or three weeks. However, the level of carcass fat of the rats fed on the 30% fat diet was slightly higher than that of either of the other groups (table 2).



Effect of protein per Calorie ratio of the diet:

Fig. 7 On the average daily Calorie intake per body weight$^{0.87}$ during three weeks.

Fig. 8 On the average daily nitrogen intake per body weight$^{0.87}$ during three weeks.

Fig. 9 On the average daily food intake during three weeks.

···●··· fat free group; —.—×—.— 10% fat group; —Δ— 30% fat group.

DISCUSSION

The reason for the apparently different effects of the high level of fat on growth in experiments 1 and 2 becomes clear when the Prot./Cal. is considered. If the level of fat in a diet is increased while the level of protein is kept constant, the Prot./Cal. will be decreased and the protein intake will fall (fig. 8). If protein is limiting for growth, an increase in the level of dietary fat will then depress the growth rate, as was observed in experiment 1. This is in agreement with the observations of Bosshardt et al., ('46), Sellers et al., ('54),

TABLE 2

Carcass analyses of rats fed on diets containing 18 mg of Prot./Cal. with different levels of fat [1]

DIETARY LEVEL OF FAT, %	0	10	30
	%	%	%
Moisture –			
2nd week	67.7 ± 0.5 [2]		67.3 ± 0.2 [2]
3rd week	66.8 ± 0.3	67.9 ± 0.4 [2]	67.3 ± 0.4
Fat –			
2nd week	10.3 ± 0.4		11.3 ± 0.2
3rd week	10.9 ± 0.7	11.0 ± 0.4	11.6 ± 0.5

[1] All values are averages for groups of 6 rats.

[2] The standard error of the mean as calculated from the formula:

$$S\bar{x} = \sqrt{\frac{\Sigma (X - \bar{x})^2}{n (n-1)}}$$

and with the observations on chicks cited in the introduction. On the other hand, if the Prot./Cal. is kept constant as in experiment 2, growth will not be retarded when the fat content of the diet is raised. The gain per unit of nitrogen consumed was not appreciably affected by the fat content of the diet in experiment 1. This is apparently true because, as can be seen from the curve relating gain per unit of nitrogen to Prot./Cal. (fig. 3), the Prot./Cal. ratios of these diets fall within the range of maximum protein efficiency. On the other hand, the gain per Calorie decreased as the fat content of the diet was increased. This effect paralleled the effect

on the growth rate (fig. 1). From figure 2 it can be seen that with lower Prot./Cal., Gain/Cal. is lower, apparently because a greater proportion of the calories is required for maintenance when the rate of gain is low.

The results obtained in experiment 2 may now be examined in more detail. The improved growth with higher Prot./Cal. ratios (fig. 1) is ascribed to the increase in protein intake. Donaldson et al., ('56), using chickens, found improved growth and improved calorie utilization until the protein level was increased above that required for maximum growth. But Gain/N (which they expressed as protein per gram gain) decreased when the protein content was increased. In the present study, the Gain/N first increased and then decreased with increasing Prot./Cal. The trend observed (fig. 3) is similar to that observed by Schreiber and Elvehjem ('55) for protein efficiency ratios. This trend can best be explained as follows: when protein intake is low, growth is low; therefore, Gain/N is low because the maintenance requirement represents a large proportion of the nitrogen intake. As the protein intake is increased, the rate of gain increases and the Gain/N increases to a maximum at the point where protein is most efficiently utilized and maintenance represents a smaller proportion of the intake. When protein intake is increased still further (fig. 8), a greater proportion of the protein is used for energy and, even though the animals continue to gain weight, Gain/N falls, and the percentage of nitrogen retained also falls (fig. 4).

The Gain/Cal. (fig. 2) follows a different pattern, and, like the growth curve (fig. 1), continues to increase with each increase in Prot./Cal. up to 60. The Gain/Cal. would be expected to increase until the point was reached at which protein intake was sufficiently high to permit maximum growth. This point would vary with the nutritive value of the protein.

Osborne, Mendel and Ferry ('19) showed that the maximum gain of body weight per gram of protein was obtained

when the food contained 7.9% of lactalbumin or 12% of casein.

Calorie intake/$W^{0.87}$ was quite constant regardless of the protein or fat content of the diet or rate of gain (fig. 7). Basal metabolic rate is generally considered to be proportional to the body weight to the 0.75 power (Brody, '45) but Hegsted and Haffenreffer ('49) suggested that 0.84 to 0.90 was more accurate for rats of body weights between 40 and 100 gm. Actually, little difference was found whether 0.75 or 0.87 was used. Fairly constant caloric intake per unit of body weight, regardless of the food composition, has been noted by several workers Smith and Carey, '23; Cowgill '28; Hill and Dansky,'50, '54; Sibbald et al., '56, '57; Sellers et al., '54; Peterson et al., '54). It is thus evident that, within physiological limits, the animals eat to meet their caloric requirement. Although caloric intake per unit of body weight is constant, if growth is limited by an inadequate protein intake, *total* caloric intake will be a function of the protein level.

Sibbald et al. ('57) indicated that percentage of nitrogen retention gradually decreased as the Prot./Cal. was increased. The lower nitrogen retention was observed with 60 mg of Prot./Cal. in our experiment. In their experiment, they used a mixture of casein, lactalbumin, DL-methionine, L-histidine and DL-threonine as the protein source while we used only casein. Their Prot./Cal. fell between 21 and 47 mg Prot./Cal. Since nitrogen intake increases as the Prot./Cal. is increased (fig. 8), percentage of nitrogen retention would decrease once the point of optimum utilization were exceeded (fig. 3). This would occur at lower Prot./Cal. with a protein of high biological value. The lower biological value of casein might lead to fairly constant nitrogen retention throughout a wide range of Prot./Cal. because a higher protein intake would be needed to satisfy the protein requirement.

At the same Prot./Cal. the group fed the high-fat diet showed somewhat greater growth, Gain/Cal. and Gain/N, but the percentage of nitrogen retention was not affected by

the fat content of the diet. Therefore, fat seems to have no effect on protein utilization itself. In the early work of Forbes et al. ('46 a, b,) some evidence that fat improved protein utilization was obtained but further studies (Forbes et al., '46 c, d and French et al., '48) showed no beneficial effect of fat. Their conclusion was that fat improved the calorie utilization only but not the protein utilization. Munro ('51) and Metta and Mitchell ('56) also concluded that there was no difference between the effects of fat and carbohydrate on protein utilization, as was mentioned in the introduction. Schreiber and Elvehjem ('55) observed that progressive substitution of fat for carbohydrate resulted in increased efficiency of protein utilization. This was concluded from the improved Gain/N of groups receiving the high fat diets. Our experiment also showed a similar effect of fat on Gain/N although the percentage of nitrogen retention was not improved. However, Gain/N represents, not protein utilization, *per se,* but calorie utilization per unit of nitrogen intake, and does not take into account the relative proportion of the nitrogen consumed which would be required for maintenance. The maintenance requirement would become less significant as the rate of gain increased, thus it would be possible to have substantial differences in Gain/N with no actual difference in the percentage of nitrogen retained until the nitrogen intake became excessive.

The results also show that fat had a beneficial effect on calorie utilization. Yet the relationship between total gain and total calorie intake remained constant whether the diets contained fat or not (fig. 4). However, if the high fat group ate more calories than the group receiving no fat, their apparent Gain/Cal. would be higher than that of the other groups. This does not mean that additional fat improves calorie utilization but that less of the calories consumed were used for maintenance when the caloric intake was greater. The intersection of the line and the abscissa in figure 5 should represent the three-week maintenance requirement

for calories of the rats at the start of the experiment. The beneficial effect of fat on Gain/Cal. is especially evident in the first week of the experiment. If the rats on diets containing fat gain more the first week although they eat more calories, this would result in a better apparent Gain/Cal. Thereafter caloric intake per body weight to the 0.87 power is almost the same for all groups. Actually the caloric intake/$W^{0.87}$ for the first week was not appreciably greater for the high-fat groups. However, since these measurements were not highly accurate with the small rats, the significance of this point remains to be examined in detail. It is conceivable that the decrease in the sucrose content of the diet makes possible a greater food intake (Harper and Spivey, '57). The lower specific dynamic effect of fat also has to be considered, but this difference seems to be too small to account for the effect observed in these experiments in which only part of the carbohydrate was replaced by fat.

In conclusion, if there is some beneficial effect of fat on calorie utilization, it would be transitory. However, protein intake and the ratio of protein to Calories are important factors affecting both calorie and nitrogen utilization.

SUMMARY

The effects of changes in the protein per Calorie ratio of the diet and in the level of dietary fat on growth, body gain per Calorie consumed and body gain per gram of nitrogen consumed have been examined using young rats fed on diets containing casein as the source of protein.

Total gain and gain per Calorie consumed increased gradually with each increase in protein per Calorie ratio up to a level of 60 mg of protein per Calorie. The optimum protein per Calorie ratio for growth and calorie utilization is probably above this when casein is the dietary protein.

The body gain per gram of nitrogen consumed increased to a maximum at between 20 and 30 mg of casein per Calorie and then decreased. This maximum, which would be de-

termined by the nutritive value of the protein, represents the point of optimum calorie utilization per unit of nitrogen consumed.

The evidence obtained indicated that the fat content of the diet influenced neither the percentage of ingested nitrogen retained nor the efficiency of calorie utilization. There did appear to be some transitory beneficial effect of additional fat in stimulating food consumption and growth during the first week of the experiment when the protein per Calorie ratio was held constant.

The average calorie intake per unit of body weight to the 0.87 power was almost constant regardless of the protein or fat content of the diet.

It has been concluded that calorie utilization and nitrogen utilization in the growing rat are not affected appreciably by the fat content of the diet but that protein to calorie ratio is an important factor to be considered in interpreting such measurements.

LITERATURE CITED

BOSSHARDT, D. K., W. PAUL, K. O'DOHERTY AND R. H. BARNES 1946 The influence of caloric intake on the growth utilization of dietary protein. J. Nutrition, *32:* 641.

BRODY, S. 1945 Bioenergetics and Growth. Reinhold Publishing Corporation, New York.

CALLOWAY, D. H., AND H. SPECTOR 1955 Nitrogen utilization during caloric restriction. I. The effect of dietary fat content. J. Nutrition, *56:* 533.

COHN, C., AND D. JOSEPH 1956 Nitrogen sparing action of neutral fat and fatty acids in the phlorhizinized rat. Proc. Soc. Exp. Biol. Med., *93:* 462.

COMBS, G. F., AND G. L. ROMOSER 1955 A new approach to poultry feed formulation. Maryland Agr. Exp. Sta. Misc. Pub. No. 226.

COWGILL, G. R. 1928 The energy factor in relation to food intake: Experiments on the dog. Am. J. Physiol., *85:* 45.

DEUEL, H. J., JR. 1948 Caloric, vitamin and mineral requirements with particular reference to protein nutrition. In "Proteins and Amino Acids in Nutrition," Edited by M. Sayhun. Reinhold Publishing Corporation, Norwood, Mass. pp. 82–131.

———— 1955 Fat as a required nutrient of the diet. Feredation Proc., *14:* 639.

DONALDSON, W. E., G. F. COMBS AND G. L. ROMOSER 1956 Studies on energy levels in poultry ration. I. The effect of calorie-protein ratio of the ration on growth, nutrient utilization and body composition of chicks. Poultry Sci., *35:* 1100.

DONALDSON, W. E., G. F. COMBS, G. L. ROMOSER AND W. C. SUPPLEE 1955 Body composition, energy intake, feed efficiency, growth rate and feather condition of growing chickens as influenced by Calorie-protein ratio of the ration. Ibid., *34:* 1190.

FORBES, E. B., R. W. SWIFT, R. F. ELLIOT AND W. H. JAMES 1946a Relation of fat to economy of food utilization. I. By the growing rat. J. Nutrition, *31:* 203.

———— 1946b Relation of fat to economy of food utilization. II. By the mature albino rat. Ibid., *31:* 213.

FORBES, E. B., R. W. SWIFT, W. H. JAMES, J. W. BRATZLER AND A. BLACK 1946c Further experiments on the relation of fat to economy of food utilization. I. By the growing albino rat. Ibid., *32:* 387.

FORBES, E. B., R. W. SWIFT, E. J. THACKER, V. R. SMITH AND C. E. FRENCH 1946d Further experiments on the relation of fat to economy of food utilization. II. By the mature albino rat. Ibid., *32:* 397.

FRENCH, C. E., A. BLACK AND R. W. SWIFT 1948 Further experiments on the relation of fat to economy of food utilization. III. Low protein intake. Ibid., *35:* 83.

HARPER, A. E., AND H. SPIVEY 1957 Effect of type of carbohydrate on protein utilization. Federation Proc., *16:* 1060.

HEGSTED, D. M., AND V. K. HAFFENREFFER 1949 Caloric intake in relation to the quality and quantity of protein in the diet. Am. J. Physiol., *157:* 141.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

HILL, F. W., AND L. M. DANSKY 1950 Studies on the protein requirements of chicks and its relation to dietary energy level. Poultry Sci., *29:* 763.

———— 1954 Studies on the energy requirement of chickens. I. The effect of dietary energy level on growth and feed consumption. Ibid., *33:* 112.

LEONG, K. C., M. L. SUNDE, H. R. BIRD AND C. A. ELVEHJEM 1955 Effect of energy: protein ratio on growth rate, efficiency, feathering, and fat deposition in chickens. Ibid., *34:* 1206.

MATTERSON, L. D., L. M. POTTER, L. D. STINSON AND E. P. SINGSEN 1955 Studies on the effect of varying protein and energy levels in poultry rations on growth and feed efficiency. Ibid., *34:* 1210.

METTA, V. C., AND H. H. MITCHELL 1956 A comparison of the biological values of dietary protein incorporated in high- and low-fat diets. J. Nutrition, *59:* 501.

MUNRO, H. N. 1951 Carbohydrate and fat as factors in protein utilization and metabolism. Physiol. Rev., *31:* 41.

OSBORNE, T. B., L. B. MENDEL AND E. L. FERRY 1919 A method of expressing numerically the growth-promoting value of proteins. J. Biol. Chem., *37:* 223.

PETERSON, D. W., C. R. GRAU AND N. F. PEEK 1954 Growth and food consumption in relation to dietary level of protein and fibrous bulk. J. Nutrition *52:* 241.

ROSENTHAL, H. L. 1952 The effect of dietary fat and caloric restriction on protein utilization. Ibid., *48:* 243.

ROSENTHAL, H. L., AND J. B. ALLISON 1956 Effects of caloric intake on nitrogen balance and organ composition of adult rats. J. Agr. Food Chem., *4:* 792.

SCHREIBER, M., AND C. A. ELVEHJEM 1955 Water restriction in nutrition studies. I. Level of fat and protein utilization. J. Nutrition, *57:* 133.

SELLERS, E. A., R. W. YOU AND N. M. MOFFAT 1954 Regulation of food consumption by caloric value of the ration in rats exposed to cold. Am. J. Physiol., *177:* 367.

SHERMAN, H. C. 1941 Chemistry of Food and Nutrition. The Macmillan Company, New York. pp. 127.

SIBBALD, I. R., R. T. BERG AND J. P. BOWLAND 1956 Digestible energy in relation to food intake and nitrogen retention in the weanling rat. J. Nutrition, *59:* 385.

SIBBALD, I. R., J. P. BOWLAND, A. R. ROBBLEE AND R. T. BERG 1957 Apparent digestible energy and nitrogen in the food of the weanling rat. Ibid., *61:* 71.

SMITH, A. H., AND E. CAREY 1923 Growth on diets high in carbohydrate and high in fat. J. Biol. Chem., *58:* 425.

SUNDE, M. L. 1956 A relationship between protein level and energy level in chick rations. Poultry Sci., *35:* 350.

SWANSON, P. P. 1951 Influence of non-protein calories on protein metabolism. Federation Proc., *10:* 660.

THOMSON, W. S. T. AND H. N. MUNRO 1955 The relationship of carbohydrate metabolism to protein metabolism. J. Nutrition, *56:* 139.

THE EFFECT OF THYROID ACTIVITY ON THE CONVERSION OF CAROTENE AND RETINENE TO VITAMIN A AND ON SERUM PROTEINS

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(Received for publication June 1957)

Johnson and Baumann ('47) reported that desiccated thyroid increased liver stores of vitamin A in rats fed carotene, but thiourea or thiouracil lowered the vitamin A reserves in the liver. These findings were confirmed by Kelley and Day ('48). Barrick, Andrews, Beeson and Harper ('48) indicated that very high doses of thiouracil inhibited the conversion of carotene to vitamin A in feeder lambs and it was also shown to be true in sheep by Bolin and Bolin ('49). Cama and Goodwin ('49) showed that desiccated thyroid increased absorption of carotene from the intestines whereas thiouracil decreased the absorption. This finding was supported by the work of Chanda, Clapham, McNaught and Owen ('51) in lactating cows and goats.

Bieri and Schultze ('51) could not demonstrate any effect of thiouracil on the vitamin A in the serum, liver and kidneys of rats fed or injected with aqueous dispersions of carotene. Arnrich and Morgan ('54), using liver storage of vitamin A as the criterion to measure the effect of thiouracil on the conversion of carotene to vitamin A, found that carotene-fed rats, rendered hypothyroid with thiouracil, stored much more vitamin A in the liver than their respective controls. Arnrich ('55) extending the studies to dogs, could not demonstrate increased storage of vitamin A from carotene in the liver of the animals treated with thiouracil. McGillivray, Thompson and Worker ('56) and Worker ('56), found that thyroidectomy or hyper-

thyroidism had no direct effect on the conversion of intravenously injected carotene to vitamin A as observed by similar blood levels and liver storage of vitamin A in treated groups. Clinical studies by earlier workers have been reviewed by Drill ('43).

In the present investigation, assuming that the conversion of carotene to vitamin A *in vivo* takes place in two stages, (a) β-carotene → retinene (Glover and Redfearn, '54) and (b) retinene → vitamin A (Glover, Goodwin and Morton, '48) the effect of hyper- and hypothyroidism on the conversion of retinene to vitamin A in rats was investigated. The fate of intravenous injections of retinene to hyper- and hypothyroid rats was studied. On account of recent conflicting reports, the effect of iodinated casein and thiourea on carotene conversion to vitamin A was also examined.

Electrophoretic analyses of sera from rats were also carried out to study the effect of iodinated casein and thiourea on the different components of serum proteins.

MATERIALS AND METHODS

Preparation of retinene for feeding and injection

The method used for preparing retinene was essentially the same as that described by Ball, Goodwin and Morton ('48), but in the present investigation vitamin A acetate,[1] after saponification, was used as the starting material instead of fish-liver oil. The reaction with manganese dioxide [2] was complete in three days, and the resulting retinene solution was chromatographed three times over 10% alumina.[3] Retinene thus obtained had an $E^{1\%}_{1cm}$ value of 1360 at 370 mμ (light petroleum). It was approximately 80% pure on the basis of the $E^{1\%}_{1cm}$ value of 1690 for crystalline retinene.

To known volumes of retinene and α-tocopherol acetate,[1] a measured quantity of arachis oil was added and the solvent

[1] Obtained from Hoffmann-La-Roche Ltd., Switzerland.

[2] B. D. H. Laboratory reagent.

[3] Alumina, specially prepared for chromatographic adsorption. Obtained from E. Merck, Germany.

removed in vacuo, so that the desired amount of retinene, equivalent to 40 μg of crystalline retinene was present in 0.1 ml of the oil which also contained 0.5 mg of α-tocopherol acetate.

The aqueous dispersion of retinene for injection was prepared by using 2% (w/v) solution of Tween 80 [4] (polyoxyethylene sorbitan monooleate) in physiological saline so that an amount equivalent to 400 μg of crystalline retinene was present in 0.4 ml.

Preparation of carotene for feeding

The oily solution of carotene for feeding was prepared as described above so that 0.1 ml of arachis oil contained 40 to 60 μg of crystalline carotene.[5] For higher dose levels of 400 and 3000 μg, the carotene was present in 0.2 ml of the oil.

For quantitative feeding of oily solutions of retinene and carotene, a micrometer syringe [6] with a blunt needle was used. Very accurate volumes for antimony trichloride colour test were also measured in the same way.

Preparation of animals and design of the experiment

Litter mates of albino rats of both sexes were used in separate groups. When the rats were 5 weeks old and had reached a weight of 25 to 40 gm, they were divided into three groups of the same sex and fed the following vitamin A-deficient diet: casein (ether extracted) 18, cornstarch 59, sugar 10, salt mixture [7] 4, refined arachis oil 8.8, cystine 0.2, plus the following vitamins per kilogram of diet: α-tocopherol acetate 100 mg, pantothenic acid 50 mg, calciferol 100 μg, menadione sodium bisulphite 10.2 mg, thiamine hydrochloride 5 mg, pyridoxine 5 mg, riboflavin 5 mg, niacinamide 50 mg, biotin 0.5 mg, folic acid 0.5 mg, inositol 100 mg, choline chloride 1.0 gm.

Rats in the control group were fed the above mentioned vitamin A-free diet. In another group, they were rendered

[4] Atlas Powder Co., Wilmington, Delaware, U. S. A.
[5] British Drug Houses Ltd., U. K.
[6] Agla, Burroughs Wellcome Ltd., U. K.
[7] Hawk and Oser, 1931.

hyperthyroid by treatment with iodinated casein [8] at a level of 0.125% of the diet. Similarly, in the third group, a hypothyroid state was induced by feeding either thiouracil [9] or thiourea [10] at levels of 0.1 and 0.2% of the diet respectively. The dosage of thiourea at 0.5% of the diet (Johnson and Baumann, '47) was found to be toxic in the experiments in which retinene and thiourea were fed. The rats were therefore treated with thiourea at a level of 0.5% of the diet for the initial three weeks of the experiment and at 0.2% level for the subsequent three weeks.

Each experiment was divided into two periods; one of about 4 weeks on vitamin A-free diet and one of two weeks during which the rats were daily dosed orally with an oily solution of retinene or crystalline carotene. Throughout the depletion period and the period of supplementation with retinene or carotene, the feeding of iodinated casein, thiouracil or thiourea was continued. In all series, a rat was considered depleted when the weighings on three consecutive days indicated that growth had virtually ceased.

For intravenous injections of retinene, the rats were treated as described above. A Tween dispersion containing an amount equivalent to 400 μg of crystalline retinene in 0.4 ml was injected into the tail vein.

Determination of vitamin A in livers and kidneys of rats fed retinene or carotene

Twenty-four hours after the last dose of retinene or carotene, the animals were anaesthetised with chloroform and killed. The livers and kidneys were separately ground with acid-washed sand and anhydrous sodium sulphate,[11] and the tissue lipid was extracted 5 times with light petroleum.[12] The combined extracts were reduced in volume at low pressure.

[8] Boots Drugs Co., U. K.

[9] Nutritional Biochemicals Corporation, U. S. A.

[10] British Drug Houses Ltd., U. K.

[11] B. D. H. Laboratory reagent.

[12] Light petroleum (b.p. 40-60°C), Obtained from Burmah-Shell, is left over $KMnO_4$, washed, dried over $CaCl_2$ and twice distilled before use.

In experiments in which retinene was administered orally or intravenously to rats, vitamin A in the livers was estimated spectrophotometrically in the whole liver extract by the antimony trichloride colour test at 620 mμ (Cama, Collins and Morton, '51). Vitamin A was also estimated by the three-point correction method of Cama et al., '51, using the absorption readings at 310, 325 and 335 mμ (light petroleum) but the results obtained are not presented in table 1 since this procedure gave only the vitamin A esters in the liver as compared to the $SbCl_3$ colour test which gave the total vitamin A (esters + alcohol).

When carotene was administered to rats, the lipid residue, obtained after removal of the solvent in vacuo from the petroleum ether extract of the livers, was saponified with ethanolic KOH. Vitamin A was estimated in the unsaponifiable portions of the liver by the antimony trichloride colour test at 620 mμ.

In all series, vitamin A in the kidneys was determined in the whole kidney extracts by the antimony trichloride colour test.

The significances of the differences between control and treated groups were statistically analysed by the "t" test of significance for paired values as given by Davies ('49).

The results were considered as significant where P was between 5 and 10% or less than 5%, whereas those which had a value for P of more than 10% were insignificant.

Analysis of serum proteins by agar electrophoresis

The procedure followed for the separation of serum proteins was essentially the same as that described by Giri ('56) and Giri and Pillai ('56). The blood was taken from the rat by cardiac puncture under mild anaesthesia and allowed to clot at 37°C for one hour. The clear serum obtained after centrifugation was used for analysis. The serum (30 μl) was applied on the agar [13] gel (0.5%) containing barbital buffer [14]

[13] B. D. H. fine powder.

[14] Barbitone sodium obtained from Bayer Co., Germany.

(pH 8.6, 0.05 ionic strength). Electrophoresis (200 volts, 5 milliamperes, 4 hrs.) was carried out at room temperature (25°C). Subsequently, the agar plates were dried and stained with Amidoschwarz 10 B.[15] The quantitative evaluation of the protein components was carried out using a Photovolt Electronic Densitometer, Model 525. Total nitrogen and non-protein nitrogen were estimated by the micro-kjeldahl method.

RESULTS

Vitamin A in livers of rats fed retinene

The results presented in table 1 indicate that male and female rats fed iodinated casein stored significantly lower amounts of vitamin A in the livers than those of the control group when fed equal amounts of retinene ($P < 0.1$, series I and $P < 0.1$, series III). When the three-point correction procedure was applied to determine vitamin A esters, the differences between the control and treated groups were found to be even more significant ($P < 0.05$ for series I and $P < 0.01$ for series III). The differences in total vitamin A levels in the livers between control and thiouracil-treated rats were not statistically significant (series II and IV).

When thiourea was used to induce hypothyroidism, the differences noted in the vitamin A contents between control and treated groups of male as well as female rats were similar; the data on male and female rats are therefore discussed together in series V. Thiourea treatment significantly increased vitamin A in the liver ($P < 0.02$).

Intravenous injections of retinene

The results of intravenous injections of retinene to control, hyper- and hypothyroid rats are presented in table 1 (series VI and VII). Rats rendered hyperthyroid accumulated as much vitamin A in the livers as those of the control group (series VI), while thiourea treatment increased vitamin A in the livers ($P < 0.05$, series VII).

[15] E. Merck, Germany.

Effect of iodinated casein, thiouracil and thiourea on vitamin A in the livers of rats (a) fed retinene in arachis oil (b) injected with Tween dispersions of retinene and (c) fed crystalline carotene in arachis oil.

SERIES NO.	DOSE	NO. OF OBSERVATIONS AND SEX	GROUP	TOTAL VITAMIN A IN THE LIVER BY $SbCl_3$ COLOUR TEST (I. U.)	MEAN DIFFERENCE (CONTROL-EXP.)	S.E.[1] DIFF.	P (PROBABILITY)
I	40 μg retinene orally for 15 days	5 M	Control	806.8	+52.6	±22.9	S*[2]
		5 M	Iod. casein-fed	754.2			
II	—Do—	7 M	Control	786.57	−32.72	±29.73	N.S.
		7 M	Thiouracil-fed	819.29			
III	—Do—	8 F	Control	701.5	+44	±18.68	S*
		8 F	Iod. casein-fed	657.5			
IV	—Do—	8 F	Control	734.75	−26.75	±42.08	N.S.
		8 F	Thiouracil-fed	761.50			
V	—Do—	8 (3M)	Control	315.63	−51.87	±16.82	S***
		8 (3M)	Thiourea-fed	367.50			
VI	400 μg retinene intravenously 24 hrs. before dissection	6 M	Control	213.0	−17.0	±11.09	N.S.
		6 M	Iod. casein-fed	230.0			
VII	—Do—	5 (4M)	Control	224.8	−23.8	± 8.3	S**
		5 (4M)	Thiourea-fed	248.6			
VIII	40 μg crystalline carotene for 14 days and 3000 μg carotene 24 hrs. before dissection	8 (3M)	Control	39.13	− 6.87	± 2.94	S**
		8 (3M)	Iod. casein-fed	46.00			
IX	60 μg crystalline carotene for 12 days and 400 μg carotene for the last three days	9 F	Control	47.00	+16.0	± 7.65	S*
		9 F	Thiourea-fed	31.00			

[1] Standard error of the difference.
[2] S* significant between 5 and 10% levels.
S** significant between 2 and 5% levels.
S*** significant between 1 and 2% levels.
N.S. not significant.

Oral administration of carotene

In series VIII (table 1), a single dose of 3,000 μg of crystalline carotene in oil was given to all rats 24 hours before dissection, after administration of 40 μg of carotene for 14 days of the supplementation period. In series IX, the dosage of carotene was increased to 60 μg per day for 12 days and three doses of 400 μg each were given to all rats during the last three days of the supplementation period.

Table 1 shows that iodinated casein-treated rats fed carotene accumulated larger amounts of vitamin A in the liver than the controls ($P < 0.05$, series VIII). In contrast, animals treated with thiourea had smaller amounts of vitamin A in the livers ($P < 0.1$, series IX).

Electrophoretic analysis of serum proteins

The percentage distribution of the serum proteins in rats given different treatments is presented in table 2. The values reported represent the average of duplicate experiments conducted under identical conditions and the results were observed to be consistent. It is apparent from the table, that in vitamin A-deficient rats, there is a significant increase in the α_2-, β_2- and γ-globulins and a significant decrease in albumin

TABLE 2

Percentage distribution of serum proteins from vitamin A-deficient, control, hyperthyroid and hypothyroid rats

GROUP	TOTAL PROTEINS	NON-PROTEIN NITROGEN [1]	ALBUMIN	GLOBULINS				
				α_1	α_2	β_1	β_2	γ
	gm %		*%*	*%*	*%*	*%*	*%*	*%*
Vitamin A deficient	6.1	3.6	54.3	4.8	5.1	3.8	20.0	12.0
Control	7.9	4.0	71.1	4.8	3.2	4.4	8.9	7.6
Iodinated casein-treated	7.9	7.2	62.8	4.8	5.1	4.0	18.2	5.1
Thiourea-treated	7.3	4.0	70.3	8.3	1.7	3.0	10.1	6.5

[1] Expressed as percentage of total nitrogen.

as compared to the control group. In iodinated casein-treated rats, the α_2- and β_2-globulins increase, but albumin is considerably lowered. Thiourea-treated rats show a remarkable increase in the α_1 component whereas the α_2 component of the globulins is considerably lowered. There is little change in the β- or γ-globulins or in the albumin fraction of thiourea-treated rats.

No appreciable change in the total serum protein concentration in the different groups of animals could be noted except in the case of vitamin A-deficient rats which show a decrease. The non-protein nitrogen is increased considerably by hyperthyroidism.

DISCUSSION

The demonstration of the conversion of vitamin A aldehyde (retinene) into vitamin A in the intestinal wall of rats by Glover et al. ('48) is of significance, for in all probability retinene may be an intermediate in the conversion of β-carotene into vitamin A. Glover and Redfearn ('54) have, in fact, shown the formation of retinene by stepwise degradation of β-carotene.

The results reported in the present studies show that the effects of iodinated casein and thiourea on rats fed retinene orally are contrary to those on rats fed carotene, on the basis of the liver reserves of vitamin A. Iodinated casein decreases but thiourea increases vitamin A in the liver, while thiouracil does not have any significant effect. When retinene was administered intravenously to hyper- and hypo-thyroid rats, there was no significant difference in the total vitamin A in the livers between the treated groups. [The "t" test of significance for paired values between hyperthyroid and hypothyroid groups (rows 12 and 14; table 1) could be carried out since the rats used in these groups were littermates of the same sex.] This clearly indicates that the thyroid activity does not have any direct effect on the conversion of intravenously administered retinene to vitamin A. Thus our observation is in

line with that of McGillivray et al. ('56) and Worker ('56), who showed that thyroid activity has little influence on the conversion of intravenously administered carotene into vitamin A. However, it is observed that the vitamin A levels in the livers of thiourea-treated groups are higher than those in the control groups (series VII; table 1). It seems probable that these rats in the control groups utilise more and hence store less of vitamin A than the treated ones during the 24-hour period.

It is evident from table 1 that the liver vitamin A reserves of carotene-fed rats are much lower than in those fed retinene. In general, the absorption of carotenoids by mammals is very poor as compared to both vitamin A and retinene and a considerable proportion of any ingested carotenoid is excreted in the feces (Krebs and Hume, '49; Gounelle, Marnay, Cheroux and Raoul, '52).

There is considerable evidence to show that the thyroid hormone has a significant role in the metabolism of carotene. Cama and Goodwin ('49), and Chanda et al. ('51) demonstrated that its effect is mainly on the intestinal absorption of carotene. The claim of Arnrich and Morgan ('54) that neither absorption, transformation nor utilisation of carotene is affected by thyroid activity was re-investigated using the criterion of liver storage of vitamin A. In case of hyperthyroidism, the liver vitamin A levels in rats are significantly high and thiourea treatment significantly lowers vitamin A in the livers (table 1). This confirms the observations reported by Johnson and Baumann ('47) and Kelley and Day ('48). We have thus to attempt a reconciliation between contradictory experiences by groups of workers employing different experimental procedures. It is important to note that the experimental procedures adopted by Arnrich and Morgan ('54) are different from those employed by previous workers. Arnrich and Morgan ('54), in fact, conclude that, in part, the disagreement found on the subject of carotene utilization in hypothyroidism is undoubtedly due to discrepancies in experimental procedures and the criteria used to measure carotene utilization.

The vitamin A reserves in the kidneys of rats fed retinene were, however, higher for hyperthyroid females than controls ($P < 0.01$) and lower for thiourea-treated animals ($P < 0.05$), which is in line with the observations of Kelley and Day ('48) on carotene. Our results also confirm the report of Moore and Sharmann ('50) who observed that males deposit more vitamin A in kidneys than females.

In vitamin A deficiency, protein synthesis is inhibited as indicated by the remarkably low level of albumin and the total serum protein value (table 2). The high value for γ-globulin is indicative of an increased production of antibodies in the system, which is characteristic of any infection. α_2-Globulin is known to increase in cases of inflammatory lesions and in other cases of tissue destruction. From the abnormally high value of α_2-globulin observed in vitamin A-deficient rats, it is reasonable to presume that the animal is in a similar state of metabolic disturbance.

Our results on hyperthyroidism (table 2) confirm the findings of Lewis, McCullagh and Clark ('44) who reported that albumin always remains low, often accompanied by an increase in α-globulins. In the case of hypothyroidism, it is observed that there is a very high increase of α_1-globulin to almost double the concentration of that in the control group but there is little change in albumin concentration. This finding is in agreement with some of the earlier observations made by Moore, Levin and Smelser ('45) and Leathem and Seeley ('47).

SUMMARY

The effect of iodinated casein, thiourea and thiouracil on retinene and carotene metabolism in rats has been investigated.

Iodinated casein decreased whereas thiourea increased the liver storage of vitamin A on feeding retinene, but thiouracil did not show any significant effect. There was no significant difference in total vitamin A in livers between the iodinated casein and thiourea groups, when retinene was injected intravenously. On feeding carotene, hyperthyroid rats stored more

and hypothyroid rats less vitamin A in the liver than the controls.

The changes occurring in the different components of serum proteins, in vitamin A-deficient, hyperthyroid and hypothyroid rats have been shown. In vitamin A deficiency a very low albumin level and a significant increase in α_2-, β_2- and γ-globulin levels are observed. In hyperthyroidism, there is a decrease in the albumin and an increase in the α_2- and β_2-globulins. In hypothyroidism an increase in the α_1-globulin is noted.

ACKNOWLEDGMENTS

Our thanks are due to Dr. M. Sirsi of the Pharmacology Laboratory for the intravenous injections of retinene and to Prof. K. V. Giri for his keen interest in this work.

LITERATURE CITED

ARNRICH, L. 1955 The effect of hypothyroidism on the metabolism of carotene in dogs. J. Nutrition, *56:* 35–49.

ARNRICH, L., AND A. F. MORGAN 1954 The utilization of carotene by hypothyroid rats. Ibid., *54:* 107–119.

BALL, S., T. W. GOODWIN AND R. A. MORTON 1948 Studies on vitamin A. 5. The preparation of retinene$_1$-vitamin A aldehyde. Biochem. J., *42:* 516–523.

BARRICK, E. R., F. N. ANDREWS, W. M. BEESON AND C. HARPER 1948 Effects of thiouracil and similar compounds on feeder lambs. J. Animal Sci., *7:* 539.

BIERI, J. G., AND M. O. SCHULTZE 1951 The utilization of solubilized aqueous carotene by normal and hypothyroid rats. Arch. Biochem. Biophys., *34:* 280–284.

BOLIN, F. M., AND D. W. BOLIN 1949 Effect of thiourea on the conversion of carotene into vitamin A. Proc. N. Dakota. Acad. Sci., *2:* 46–48.

CAMA, H. R., F. D. COLLINS AND R. A. MORTON 1951 Studies in vitamin A. 17. Spectroscopic properties of all-*trans*-vitamin A and vitamin A acetate. Analysis of liver oils. Biochem. J., *50:* 48–60.

CAMA, H. R., AND T. W. GOODWIN 1949 Studies in vitamin A. 9. The role of the thyroid in carotene and vitamin A metabolism. Ibid., *45:* 236–241.

CHANDA, R., H. M. CLAPHAM, M. L. MCNAUGHT AND E. C. OWEN 1951 The digestibility of carotene by the cow and the goat as affected by thyroxine and thiouracil. Ibid., *50:* 95–99.

DAVIES, O. L. 1949 Statistical methods in research and production. 2nd ed. (revised) Edinburgh and London: Oliver and Boyd, P. 57.

DRILL, V. A. 1943 Interrelations between thyroid function and vitamin metabolism. Physiol. Rev., *23:* 355–379.

GIRI, K. V. 1956 Agar electrophoresis, Part 1. A simple clinical method for the analysis of serum proteins. J. Indian Institute of Science, *38:* 190–199.

GIRI, K. V. AND N. C. PILLAI 1956 Multiple haemoglobins in the blood of animals. Nature, *178:* 1057.

GLOVER, J., T. W. GOODWIN AND R. A. MORTON 1948 Studies in vitamin A. 6. Conversion *in vivo* of vitamin A aldehyde (retinene$_1$) to vitamin A$_1$. Biochem. J., *43:* 109–114.

GLOVER, J., AND E. R. REDFEARN 1954 The mechanism of the transformation of β-carotene into vitamin A *in vivo*. Ibid., *58:* XV–XVI.

GOUNELLE, H., C. MARNAY, R. CHEROUX AND Y. RAOUL 1952 Absorption intestinale du retinene et sa transformation rapide en vitamin A chez l'homme Compt. rend. soc. biol., *146:* 523–525.

HAWK, P. B., AND B. L. OSER 1931 A modification of the Osborne-Mendel salt mixture. Science, *74:* 369.

JOHNSON, R. M., AND C. A. BAUMANN 1947 The effect of the thyroid on the conversion of carotene into vitamin A. J. Biol. Chem., *171:* 513–521.

KELLEY, B., AND H. G. DAY 1948 Thiouracil and the conversion of carotene to vitamin A in the rat. Ibid., *175:* 863–866.

KREBS, H. A., AND E. M. HUME 1949 Vitamin A requirement of human adults. Sp. Rep. Ser. Med. Res. Council, London, No. 264.

LEATHEM, J. H., AND R. D. SEELEY 1947 Plasma and liver protein concentrations of rats fed thiouracil. Am. J. Physiol., *149:* 561–564.

LEWIS, L. A., E. P. MCCULLAGH AND J. CLARK 1944 Electrophoretic analysis of plasma proteins in hyperthyroidism and hypothyroidism. Am. J. Med. Sci., *208:* 727–735.

MCGILLIVRAY, W. A., S. Y. THOMPSON AND N. A. WORKER 1956 Further studies on the metabolism of intravenously administered aqueous dispersions of carotenoid pigments. Brit. J. Nutrition, *10:* 126–134.

MOORE, D. H., L. LEVIN AND G. K. SMELSER 1945 Electrophoretic and salt fractionation of the serum proteins of normal and hypothyroid rats. J. Biol. Chem., *157:* 723–730.

MOORE, T., AND I. M. SHARMANN 1950 Vitamin A in the kidneys of male and female rats. Biochem. J., *47:* Xliii–Xliv.

WORKER, N. A. 1956 The effect of the thyroid on the conversion of intravenously administered aqueous dispersions of carotene to vitamin A in the rat. J. Nutrition., *60:* 447–454.

COPPER CONTENTS OF CITRATED WHOLE BLOOD AND PLASMA OF CATTLE [1]

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(Received for publication July 8, 1957)

When the evaluation of the copper status of cattle was undertaken in Oregon in 1950, it was thought desirable to utilize blood plasma instead of whole blood in order to minimize the interference of iron with the determination of copper. Citrated blood obtained from cattle presumed to be normal showed approximately 1 μg Cu/ml of either plasma or whole blood. As studies progressed in marginal areas, large numbers of values of the order of 0.1 to 0.2 μg Cu/ml of plasma were obtained without correspondingly severe symptoms of copper deficiency anticipated from studies previously reported by other workers. (Cunningham, '45; Jamieson and Allcroft, '50; Marston, '52). Since plasmas from whole bloods having in the vicinity of 0.30 μg Cu/ml were found to contain only about 0.13 μg Cu/ml, it was felt worth while to reinvestigate this relationship over the entire range of copper values. It is our purpose in this paper to report in some detail studies covering 805 citrated whole bloods and their corresponding plasmas.

MATERIALS AND METHODS

The samples used in this study were obtained by drawing blood from the jugular vein. Saturated sodium citrate at the rate of 1.25 ml/100 ml of blood was used as an anticoagulant. Plasma was obtained by centrifuging. All samples were kept

[1] Technical Paper no. 1060, Oregon Agricultural Experiment Station.

on ice or under refrigeration from the time of bleeding until analyses were completed. Ten-milliliter samples of plasma or whole blood were wet-ashed (Eden and Green, '40) with sulfuric, perchloric and nitric acids. Copper was determined spectrophotometrically, using sodium diethyldithiocarbamate (Eden and Green, '40) followed by extraction with CCl_4. Blanks for contamination from manipulation, laboratory surroundings, glassware, distilled water and reagents were run with each series of analyses.

Photometric measurements were made with a Coleman Model 11 spectrophotometer at 440 millimicrons set at 100% transmission with the solvent. Extinction values of the blanks were determined and subsequently subtracted from those of the samples, thus providing a record of the overall magnitude of contamination from all sources.

RESULTS AND DISCUSSION

The results obtained are summarized in table 1. These samples represent various exploratory studies followed by more intensive sampling in problem areas. They are selected only to the extent that this study is confined to samples on which copper values were available for both citrated whole bloods and their corresponding plasmas. The lower copper values represented by some 200 samples are from animals and areas in which symptoms of copper deficiency, such as bleached hair coats, scouring and fragile bones have been known for many years. Most of the values above 1.0 μg/ml come from an area in which copper supplementation is known to have been practiced.

The dietary spectrum represented by these data varies from forages containing 1 to 2 p.p.m. to 5–10 p.p.m. of copper (air-dry basis). Molybdenum varies from less than 1 p.p.m. to over 10 p.p.m. The sulfate pattern reflects soils varying from extreme sulfur deficiency to those containing excessive amounts of sulfates. Deficiency symptoms may therefore result from simple copper deficiency or be complicated by molybdenum-sulfate and possibly other relationships.

The lowest blood copper values originated mostly from beef cattle grazed on an alkaline phosphorus-deficient reclaimed lake bed (Klamath) producing forages with 1 to 3 p.p.m. of copper and frequently containing some 2 to 10 p.p.m. or more of molybdenum. In this and an adjacent peaty area scouring is a serious problem. In addition, the peaty area has a long history of fragile bones in lambs and young beef cattle. A considerable number of values in the vicinity of 0.20 μg Cu/ml

TABLE 1

Copper contents of whole bloods and plasmas

PLASMA COPPER INTERVALS	NO. OF SAMPLES	COPPER		BLOOD MINUS PLASMA
		Blood	Plasma	
μg/ml		*μg/ml*	*μg/ml*	*μg/ml*
0.000 – 0.099	32	0.222	0.072	0.150 ± 0.012
0.100 – 0.199	125	0.319	0.142	0.177 ± 0.007
0.200 – 0.299	66	0.410	0.240	0.170 ± 0.010
0.300 – 0.399	51	0.507	0.352	0.155 ± 0.009
0.400 – 0.499	40	0.582	0.443	0.139 ± 0.007
0.500 – 0.599	60	0.666	0.550	0.116 ± 0.008
0.600 – 0.699	123	0.774	0.651	0.123 ± 0.006
0.700 – 0.799	92	0.822	0.740	0.082 ± 0.006
0.800 – 0.899	107	0.905	0.847	0.058 ± 0.006
0.900 – 0.999	63	0.973	0.942	0.031 ± 0.009
1.000 – 1.099	33	1.027	1.052	−0.025 ± 0.014
1.100 – 1.199	30	1.108	1.158	−0.050 ± 0.010
1.200 – 1.299	11	1.169	1.251	−0.082 ± 0.021
1.300 – 1.399	4	1.192	1.346	−0.154 ± 0.064
1.400 – 1.499	3	1.274	1.441	−0.167 ± 0.181
1.500 – 1.599	1	1.390	1.570	−0.180

plasma came from a marshy (lower Columbia) area in which forages contain 5 to 10 p.p.m. of copper together with 3 to 10 p.p.m. of molybdenum. Here scouring in young beef cattle is a serious problem. Appropriate copper supplementation restores blood copper levels and alleviates deficiency symptoms in both areas.

The significance of marginal to low blood copper levels deserves critical comment. Values less than 0.70 μg Cu/ml whole blood are widely accepted as evidence of impaired copper status (Marston, '52). It does not follow that this marks

the point below which clear-cut deficiency symptoms become evident. This is particularly true of anemia. Under conditions prevailing in Oregon, scattered marginal to low hemoglobin values have not fallen into any discernible dietary or regional pattern. This statement also applies to red cell volume.

Plasma copper values are arranged in intervals as shown in column 1. The average plasma values for each interval are shown in column 4. In column 3 are given the averages of the whole bloods from which these plasmas were obtained. Column 5 contains the differences between the whole blood and plasma average for each interval, including standard error ($s_{\bar{x}}$) of these differences.

For clarity these data are presented graphically in figure 1. Plasma intervals are plotted as ordinates. The differences shown in column 5 of table 1 are plotted as abscissas. It will be noted that only in the vicinity 1.0 μg/ml are copper values for citrated whole blood and plasma approximately equal. At higher concentrations whole bloods appear to contain less copper than do their respective plasmas. At lower concentrations, whole bloods contain more copper than their corresponding plasmas. For citrated whole bloods containing an average of 0.30 μg/Cu/ml, the corresponding plasmas contain 0.13 μg/ml. It should be emphasized that such an average difference assumes considerable importance in the evaluation of copper status in marginal to deficient areas.

The distribution of copper between erythrocytes and plasma in presumably normal cattle and man is of considerable interest. Cartwright ('50) cites values which indicate that in human blood the copper content of the cells is about 65% of that of the plasma. This difference is reflected in the investigations of Wintrobe et al. ('53) in which 63 human adults showed whole blood values averaging 0.98 μg/ml, as against plasma values averaging 1.09 μg/ml. It should be emphasized, however, that available data on man probably do not cover the wide spectrum of dietary copper environments included in figure 1. It is conceivable that the data on man are largely

confined to a copper environment analogous to that of cattle showing plasma values of 1.0 μg Cu/ml or more.

The recent work of Beck ('56) emphasizes the need for comparative studies because of the suggestion that ruminants may be somewhat unique in lacking mechanisms restricting

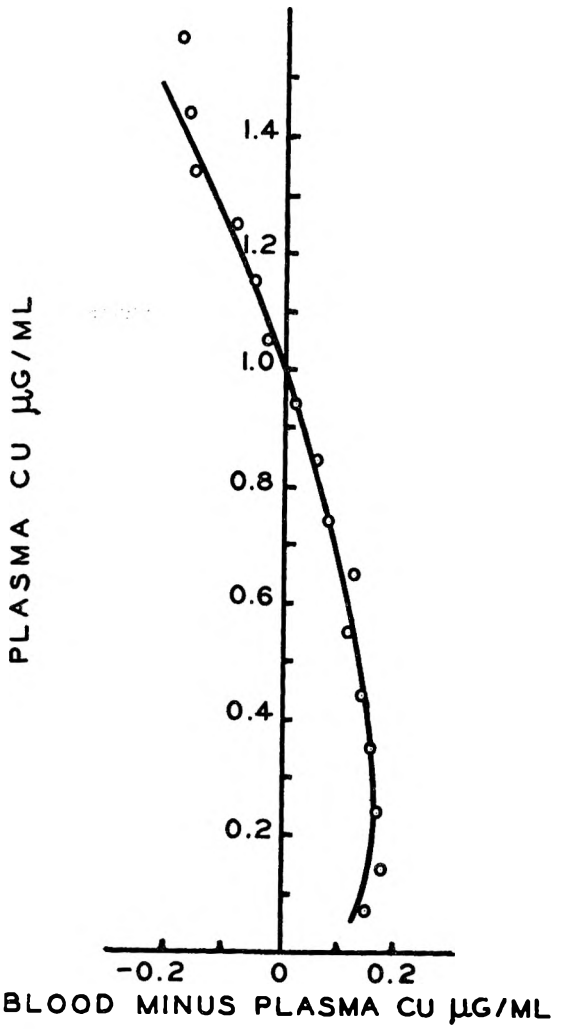

Fig. 1 Relationship between copper in whole blood and in plasma. Plasma intervals are plotted as ordinates. Abscissas represent average differences between whole blood and plasma.

the absorption and storage of copper. The extent to which such mechanisms are lacking complicates the precise definition of "normal" copper status in ruminants.

SUMMARY AND CONCLUSIONS

Data are presented on the copper contents of 805 samples of citrated whole blood and plasma from cattle. The range in copper contents covers variations in copper status extending from extreme deficiency states to more than normal intakes. Whole blood and plasma of cattle contain approximately equal concentrations of copper only in the vicinity of 1 μg/ml. Evaluation of copper status from blood analyses requires appropriate allowance for differences in the copper contents of whole blood and plasma.

LITERATURE CITED

BECK, A. B. 1956 The copper content of liver and blood of some vertebrates. Aust. Jour. Zool., *4:* 1–18.

CARTWRIGHT, G. E. 1950 Copper metabolism in human subjects, *in* Copper Metabolism: A Symposium on Animal, Plant and Soil Relationship. Edited by W. D. McElroy and Bently Glass, Johns Hopkins University Press, Baltimore, Md., page 274–314.

CUNNINGHAM, I. V. 1945 Copper deficiency in cattle and sheep on peat lands. N. Z. J. of Sci. and Tech., *27:* 381–396.

EDEN, A., AND H. H. GREEN 1940 Microdetermination of copper in biological materials. Biochem. J., *34:* 1202–1208.

JAMIESON, S., AND R. ALLCROFT 1950 Copper pine of calves. British J. Nutrition, *4:* 16–31.

MARSTON, H. R. 1952 Cobalt, copper and molybdenum in the nutrition of animals and plants. Physiol. Rev., *32:* 66–121.

WINDROBE, M. M., G. E. CARTWRIGHT AND C. J. GUBLER 1953 Studies on the function and metabolism of copper. J. Nutrition., *50:* 395–419.

EFFECT OF PARTIAL PANTOTHENIC ACID DEFICIENCY ON REPRODUCTIVE PERFORMANCE OF THE RAT[1]

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(Received for publication July 12, 1957)

INTRODUCTION

Results of experiments with several species indicate that pantothenic acid (PA) plays a role in the process of reproduction. Bauernfeind and Norris ('39) observed that eggs from hens fed a PA-deficient diet had a low hatchability, namely 15.8% as compared with 63.9% in eggs from control birds. In swine (Ullrey et al., '55), severe PA deficiency depressed or prolonged the estrous cycle of gilts and no litters were produced. Mild deficiency resulted in a decreased number of pigs weaned per litter, and signs of PA deficiency, such as tremor and intestinal bleeding appeared in some young. In female rats maintained on PA-deficient diets, smaller litters, uterine resorption, stillbirths, as well as malformations and underdevelopment of young have been observed (Jukes, '40; Nelson and Evans, '46; Lefebvres, '54; Giroud et al., '54; Everson et al., '54; Nelson et al., '57). Failure of female rats to rear their offspring has also been noticed by Sure ('41) and Giroud et al. ('54). Finally, a retarded development of sex organs in animals fed a PA-deficient diet was reported for both rats (Ashburn, '40, and Figge and Allen, '42) and mice (Melampy and Cavazos, '54). On the other hand, in rats, the forti-

[1] This study was supported by a grant-in-aid from the Nutrition Foundation, Inc.

fication of the regular laboratory chow with large amounts of PA resulted in 21 to 27% increase in litter size (Taylor et al., '34).

Most of the experiments with rats were conducted with extremely deficient diets and the rations were fed for only a short time during or before the gestation period. It was decided, therefore, to study the influence of chronic feeding of suboptimal amounts of PA on the reproductive performance of the rat. In view of the reported possible adverse effect of PA on male gonads (Ashburn, '40; Melampy and Cavazos, '54) both male and female rats were fed the experimental diets.

EXPERIMENTAL

Forty-five male and forty-five female albino rats of the Sprague-Dawley strain with an average weight of 45 to 50 gm were used in this study. The animals were housed in individual metal, screen-bottom cages in an air-conditioned room maintained at 70 to 72°F. and 45% relative humidity. In order to deprive the animals of possible PA stores, a PA-deficient diet was fed for the first 4 weeks. After this depletion period, both males and females were divided into three groups, each consisting of 15 animals. The diets of the groups were supplemented with 0.2, 0.5 and 2.0 mg of calcium pantothenate (CaP) per 100 gm of ration respectively. After the 28th day, the group on the 0.5 mg level was subdivided as follows: 7 males and 8 females were fed a diet with 0.8 mg CaP per 100 gm, the remaining animals being continued on the original 0.5 mg level.

The PA-deficient diet consisted, in percent, of vitamin-free casein, 25;[2] salts IV,[3] 4; corn oil, 5; choline chloride, 0.2; cystine, 0.2 and sucrose 65.6. Vitamins were supplied in milligrams per 100 gm of diet as follows: thiamine, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 10.0; *i*-inositol, 20; *p*-aminobenzoic acid, 20.0; folic acid, 0.1; biotin, 0.1; mena-

[2] Labco, Borden Company.
[3] Hegsted et al., '41.

dione, 1.0; and α-tocopherol acetate, 10.0. Vitamins A and D were supplied in the form of halibut oil and Drisdol.[4]

After 90 days of the experiment, the animals were considered mature (Farris and Griffith, '49) and mating experiments were started. In most cases, one male was housed with two females for a period of 7 days. Afterwards, each animal was returned to its cage. As the length of the estral cycle in the rat is about 4 days (Long and Evans, '22), a mating period of one week was thought sufficient to insure fertilization of the females (table 1).

TABLE 1

Arrangement of matings[1] *and number of litters*

CA-PANTOTHENATE PER 100 GM OF DIET		NUMBER OF		CA-PANTOTHENATE PER 100 GM OF DIET		NUMBER OF	
Females	Males	Matings	Litters	Females	Males	Matings	Litters
mg	*mg*			*mg*	*mg*		
0.2	0.2	4	0	0.8	0.2	2	0
0.2	0.5	2	0	0.8	0.5	9	6
0.2	0.8	2	0	0.8	0.8	2	2
0.2	2.0	21	5	0.8	2.0	2	0
	Total	29	5			15	8
0.5	0.2	2	0	2.0	0.2	8	1
0.5	0.5	2	0	2.0	0.5	12	6
0.5	0.8	4	2	2.0	0.8	6	1
0.5	2.0	9	6	2.0	2.0	6	3
	Total	17	8			32	11

[1] Refers to mating periods described on page 593.

The diet used during the mating period was that of the animal receiving the lower CaP concentration. For example, a male originally on the 0.8 mg level and mated with females on the 0.5 mg level, was fed the 0.5 mg level diet during the mating period. After the mating period each animal was fed its original diet.

The weights of the females were recorded and one week before expected parturition, the animals were transferred to

[4] Winthrop-Stearns, Inc.

special cages with smaller mesh screen on the bottom. At birth, the litter size and the average weight of the young were also recorded. Litters were weaned after 21 days and the females remated after a recovery period of one week. At the end of the experiments all animals were sacrificed, and ovaries and testes preserved in buffered 10% formalin for subsequent histological examination.

RESULTS AND DISCUSSION

A total of 93 matings was recorded. In an effort to determine the fertility of animals on the lowest level of vitamin intake, these animals were mated repeatedly with partners on the highest level of intake, which was considered to be most favorable for fertility. Both males and females on the 0.2 mg CaP level showed poor fertility; from 16 matings the males were successful only once and the litter consisted of only one young. In 29 matings, the females of this group gave birth to 5 litters. Both males and females on the 0.5 mg CaP level showed higher fertility; the males especially did not differ significantly from the groups on still higher levels of intake.

In about 25% of the cases of pregnancies on the 0.2 mg and 0.5 mg CaP levels, resorptions of young could be observed as judged from initial considerable increase in weight and volume of the females followed by a stationary or declining body weight. Nelson and Evans ('46), Jukes ('40), and Lefebvres ('54) also noticed the resorption of young in the PA-deficient female rats.

The PA content of the diet seemed to affect both the size and weight of litters. Nelson and Evans ('46) noted that females fed a PA-deficient diet from the first day of pregnancy gave birth to smaller and lighter litters. Everson et al. ('54) made the same observation with a PA-deficient diet fed during a period which began one week before mating and ended with parturition. Our results, summarized in table 2, show the same tendency: the females on lower CaP levels had smaller litters with lighter young. Most of the litters on the highest PA level consisted of more than 10 young, the highest number

TABLE 2

Influence of calcium pantothenate (CaP) in the diet of female rats on litter size and litter weight

CAP/ 100 GM OF DIET	NUMBER OF YOUNG PER LITTER													
	1	3	5	6	7	8	9	10	11	12	13	14	16	17
mg														
0.2		6.0[1]		5.7	5.4	5.1			5.4					
0.5			6.6		5.6			5.3		5.1		4.7		
					5.9			6.1		4.9				
0.8		6.0				6.1	5.9	5.9		5.0			5.8	
							6.2	5.2						
2.0	7.0					5.4			5.6	5.7	6.2	6.4		5.2
									5.3	6.3	5.5			
									4.9					

[1] Average weight of young at birth in grams.

in one litter being 17. The diet of the males, with the exception of those on the lowest PA level, did not seem to influence either the size or the weight of the litters.

Numerous difficulties were encountered in an attempt to rear the litters; in most cases the young, even when seemingly born normal, were not observed suckling and usually died or were eaten by the mothers during the first three days. Out of 32 litters, only 6 were reared. Similar difficulties in rearing the young born to mothers on PA-deficient diets have been reported and Sure ('41) attributed the failure of lactation to the lack of a special lactation factor. At the termination of this experiment, 6 females and 4 males previously fed a diet containing 10.0 mg CaP per 100 gm were mated. From 10 matings, 6 litters were obtained and 5 of them were successfully weaned. It seems, therefore, that the PA content of the diet plays a role in this lactation failure. It is probable that some other factors may exert a certain "co-influence," as Sure ('41) did not notice any improvements in lactation after fortifying the diet with 100 mg PA per animal per day.

In agreement with the observation of Lefebvres ('54) and Nelson et al. ('57), deformities in young born to females on low PA levels were noticed. In two cases, encephaly and anophthalmy were recorded. A litter born to a female on the 0.5 mg

CaP level showed marked underdevelopment, slow growth and retarded hair growth. The young did not open their eyes until the 28th day, as compared with 14 to 16 days in normal animals (Farris and Griffith, '49). Because of the failure of lactation already mentioned few litters survived to be weaned.

Histological examination revealed a difference in effect of chronic, partial PA deficiency on the gonads. The ovaries showed practically no pathological changes; the germ cells developed in normal numbers and numerous corpora lutea were found regardless of the diets fed. On the other hand, the testes of animals on the lowest vitamin level exhibited marked damage. The germ layer in the seminal tubules was partially broken down filling the lumen and practically no spermiogenesis was noted. Some degree of damage in the seminal tubules was noted also in animals on the two next higher vitamin levels; the spermiogenesis was, however, normal in both these groups. At the level of 2.0 mg CaP per 100 gm diet very little damage was noted and abundant sperm were found.

No definite explanation for the observed irregularities in reproductive performance of the PA-deficient females can be offered at this time. Voris et al. ('42) observed that PA deficiency depressed the appetite in the rat. Under extreme conditions this could perhaps lead to pronounced undernutrition with some disturbance in reproduction. Nelson and Evans ('46), using the paired-feeding technique, found, however, that the lowered food intake alone cannot account for the observed irregularities, since their pair-fed controls did not show any abnormal features.

The metabolically active form of pantothenic acid, coenzyme A, has been reported to be involved in the synthesis of steroid hormones (Klein and Lipmann, '53). As many phases of the reproductive process are governed by this group of compounds, PA deficiency might result in hormonal imbalance or deficiency, which could finally manifest itself in disturbed reproductive function. This suggestion seems to be supported by the findings of Braekkan ('55) that the ovaries of a species of fish, *Thunnus thynnus,* at a certain stage of the sexual cycle

contain up to 245 μg of PA per gram of fresh tissue, which seems to be the highest PA content ever reported for any tissue. Little is known about the PA content of gonads in the different species at various stages of the sexual cycle. It cannot be excluded, however, that these tissues do require a high pantothenic acid supply for normal functioning, at some stage at least, and that a deficiency of this vitamin at this particular stage would interfere with normal functioning of the glands. It can be recalled in this connection, that other steroid-producing glands, namely the adrenals, are susceptible to PA deficiency (Daft and Sebrell, '39; Daft et al., '40; Cowgill et al., '52) and that their function is impaired at an early stage of the deprivation (Deane and McKibbin, '46).

The impairment of adrenal activity itself might contribute to observed disturbances in reproduction. Adrenalectomy usually leads to interruption of pregnancy (Britton and Kline, '36) and hypotrophy of the gonads. Parkes ('45) reviewing the literature on this subject, came to the conclusion that the effect of adrenalectomy on the ovary and on pregnancy is a result of a "generally impaired health," rather than the lack of any specific gonadergic substance produced by the adrenals. The report by Cupps ('55) that certain adrenal steroids were able to restore reproduction in adrenalectomized female rats shows that this problem is not as yet settled.

SUMMARY

The effect of long-term feeding of suboptimal amounts of pantothenic acid on the reproductive performance of the rat was investigated. Male and female weanling albino rats were fed a pantothenic acid-deficient diet for 4 weeks to deplete them of the vitamin stores and then they were supplied with 0.2, 0.5, 0.8 and 2.0 mg of calcium pantothenate per 100 gm of diet. Serious anatomical and functional impairments of testes with resulting loss of fertility was noted in male rats receiving the lowest vitamin supplement. In female rats, decreased fertility, litter resorption and malformation, and

retarded development of young were observed on the two lowest vitamin levels, without any significant concomitant histological changes in the ovaries.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. J. Floody of Hoffmann-LaRoche, Inc. and Dr. N. S. Ritter of Merck Sharp & Dohme for the generous supply of vitamins.

LITERATURE CITED

ASHBURN, L. L. 1940 The effect of administration of pantothenic acid on the histopathology of the filtrate factor deficiency state in rats. U. S. Pub. Health Rep., *55:* 1337.

BAUERNFEIND, J. C., AND L. C. NORRIS 1939 The antidermatosis vitamin required for reproduction in the domestic fowl. Science, *89:* 416.

BRAEKKAN, O. R. 1955 Role of pantothenic acid in the reproduction cycle of ovaries in fish. Nature, *176:* 598.

BRITTON, S. W., AND R. F. KLINE 1936 Relation of the adrenal cortex to reproduction and lactation. Am. J. Physiol., *115:* 627.

COWGILL, G. R., R. W. WINTERS, R. B. SCHULTZ AND W. A. KREHL 1952 Pantothenic acid deficiency and the adrenals: Some recent experiments and their interpretation. Internat. Ztschr. Vitaminforsch., *23:* 275.

CUPPS, P. T. 1955 The ability of certain adrenal steroids to restore reproduction in adrenalectomized female rats. Endocrinology, *57:* 1.

DAFT, F. S., AND W. H. SEBRELL 1939 Hemorrhagic adrenal necrosis in rats on deficient diets. U. S. Publ. Health Rep., *54:* 2247

DAFT, F. S., W. H. SEBRELL, S. H. BABCOCK, JR. AND T. H. JUKES 1940 Effect of synthetic pantothenic acid on adrenal hemorrhage, atrophy, and necrosis in rats. Ibid., *55:* 1333.

DEANE, H. W., AND J. M. MCKIBBIN 1946 The chemical cytology of the rat's adrenal cortex in pantothenic acid deficiency. Endocrinology, *38:* 385.

EVERSON, G., L. NORTHROP, N. Y. CHUNG, R. GETTY AND C. PUDELKEWICZ 1954 Effect of varying the intake of calcium pantothenate of rats during pregnancy. J. Nutrition, *53:* 341.

FARRIS, E. J., AND J. Q. GRIFFITH, JR. 1949 The rat in laboratory investigation. J. B. Lippincott Co., Philadelphia.

FIGGE, F. H. J., AND E. ALLEN 1942 Genital atrophy during pantothenic acid deficiency and responses to gonadotropic and estrogenic hormones. Endocrinology, *30:* 1028.

GIROUD, A., G. LEVY AND J. LEFEBVRES 1954 Recherches sur le taux de l'acide pantothenique chez les meres et les foetus normaux et chez les meres carencees. Internat. Ztschr. Vitaminforsch., *25:* 148.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

JUKES, T. H. 1940 Reproduction in rats on synthetic B-complex supplement. Proc. Soc. Exp. Biol. Med., *45:* 625.

KLEIN, H. P., AND F. LIPMANN 1953 The relationship of coenzyme A to lipid synthesis. II. Experiments with rat liver. J. Biol. Chem., *203:* 101.

LEFEBVRES, J. 1954 Influence d'une deficience pantothenique legere sur les resultats de la gestation chez la ratte. C. R. Acad. Sci., *238:* 2123.

LONG, J. A., AND H. N. EVANS 1922 The Oestrous Cycle in the Rat and its Associated Phenomena. Memoirs University of California, *6*.

MELAMPY, R. M., AND L. F. CAVAZOS 1954 Effects of pantothenate deficiency on mouse seminal vesicles. Federation Proc., *13:* 98.

NELSON, M. M., AND H. M. EVANS 1946 Pantothenic acid deficiency and reproduction in the rat. J. Nutrition, *31:* 497.

NELSON, M. M., H. V. WRIGHT, C. D. C. BAIRD AND H. M. EVANS 1957 Teratogenic effects of pantothenic acid deficiency in the rat. Ibid., *62:* 395.

PARKES, A. S. 1945 The adrenal-gonad relationship. Physiol. Rev., *25:* 203.

SURE, B. 1941 Dietary requirements for fertility and lactation. XXIX. The existence of a new dietary factor essential for lactation. J. Nutrition, *22:* 499.

TAYLOR, A., D. PENNINGTON AND J. THACKER 1943 The effect of high levels of pantothenic acid on reproduction in the rat and mouse. Ibid., *25:* 389.

ULLREY, D. E., D. E. BECKER, S. W. TERRILL AND R. A. NOTZOLD 1955 Dietary levels of pantothenic acid and reproductive performance of female swine. Ibid., *57:* 401.

VORIS, L., A. BLACK, R. W. SWIFT AND C. E. FRENCH 1942 Thiamine, riboflavin, pyridoxine and pantothenate deficiencies as affecting the appetite and growth of the albino rat. Ibid., *23:* 555.

EFFECT OF ASCORBIC ACID IN PANTOTHENIC ACID DEFICIENCY[1]

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(Received for publication July 16, 1957)

INTRODUCTION

Several authors have reported that the inclusion of ascorbic acid (Daft, '51; Daft and Schwartz, '52; Everson et al., '54) or of related compounds (Hundley and Ing, '53) in a pantothenic acid (PA)-deficient diet alleviates the signs of PA deficiency. Similar suppression of the PA-deficiency syndrome has been observed with deficient diets supplemented with antibiotics (Lih and Baumann, '51; Sauberlich, '52; Guggenheim et al., '54). As the antibiotics are believed to act by inducing changes in intestinal microflora (Johansson et al., '53), it was decided to study whether ascorbic acid acts through the same mechanism. A high dose of sulfathiazole was used to suppress the growth of intestinal micro-organisms. The effect of antibiotics in preventing the PA-deficiency syndrome was tested with a new antibiotic, hygromycin.[2] The properties and therapeutic spectrum of this drug were described by Pittenger et al. ('53) and by Mann et al. ('53). Hygromycin is a broad-spectrum antibiotic and in preliminary investigations it showed some growth-promoting properties.

EXPERIMENTAL

Two series of experiments were conducted, using male weanling rats of the Sprague-Dawley strain with average initial

[1] This study was supported by a grant-in-aid from The National Vitamin Foundation, Inc.

[2] Kindly supplied by Dr. O. K. Behrens, the Eli Lilly Research Laboratories.

weights of about 45 gm. In the first series, each of the 5 groups consisted of 8 animals; in the second series, 10 animals for each of the 6 groups were used. The animals were housed in individual metal screen-bottom cages in an animal room maintained at 70°F and 40 to 45% relative humidity.

Food and water were supplied ad libitum and the rats were weighed at regular intervals. The first series was terminated after 52 days, the second series after 31 days.

The composition of the control PA-deficient diet was as follows: vitamin-free casein, 25%;[3] corn oil, 5%; sucrose, 65.6%; mineral salts mixture, 4%;[4] choline Cl, 0.2%; and cystine, 0.2%. Vitamins were supplied in milligrams per 100 gm of diet as follows: thiamine, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 10.0; *i*-inositol, 20; *p*-aminobenzoic acid, 20.0; folic acid, 0.1; biotin, 0.1; menadione, 1.0; and α-tocopherol acetate, 10.0. Vitamins A and D were supplied in the form of halibut oil and Drisdol.[5] The experimental rations were prepared by suitable modifications of the control diet; 2% of the sucrose was replaced by the weight equivalent of either ascorbic acid or citric acid or phthalylsulfathiazole (sulfathiazole). In one diet 4% of sucrose was replaced by 2% ascorbic acid plus 2% sulfathiazole, and the hygromycin diet was prepared by supplementing the control diet by 100 mg hygromycin per kilogram of diet in the first series and 200 mg hygromycin per kilogram of diet in the second series. The animals which died during the experiment and those that were sacrificed at the end of the experiment were autopsied, their adrenals weighed, and together with testes, preserved in buffered formaldehyde for histological examination. Serum protein distribution was studied with the help of paper electrophoresis using Spinco-Durrum cells,[6] barbiturate buffer of pH 8.6, ionic strength 0.075, constant current of 10 mA per cell, and 16-hour runs. The strips were stained in bromphenol blue and evaluated in Spinco Analytrol.

[3] Labco, Borden Company.
[4] Hegsted et al., '41.
[5] Winthrop-Stearns, Inc.
[6] Beckman, Spinco Division, Palo Alto, California.

RESULTS

The weight gains computed separately for the first 10 days of the experiment and for the following three weeks are summarized in table 1. In the first series, only the data for the first 31 days are treated statistically, because after this period, an appreciable number of animals succumbed, especially in the group receiving ascorbic acid plus sulfathiazole.

During the initial 10 days the animals grew quite rapidly, gaining daily from 2.2 gm in the group fed ascorbic acid plus sulfathiazole, to 4.1 gm in the group receiving the hygromycin supplement. After this period the growth rate declined and the group fed ascorbic acid plus sulfathiazole in the first series even lost some weight. Only the groups fed ascorbic acid or hygromycin showed more substantial weight gains, ranging from 20 to over 40 gm for the following three weeks. During the final 20 days of experiment the group receiving ascorbic acid continued to gain weight, in contrast to the group receiving hygromycin whose growth rate declined toward the end of the experiment.

At the end of about three weeks, external signs of pantothenic acid deficiency, such as thinning of the fur, porphyrine whiskers, and arched back were noted in some animals in the groups receiving ascorbic acid plus sulfathiazole. During the following week, these deficiency signs appeared in a few animals in all of the other groups with the exception of the ascorbic acid and the hygromycin groups. After about 45 days, even in these two groups a few animals showed the deficiency signs.

Upon autopsy some differences between the individual groups with respect to the condition of certain vital organs were noticed. In the second series, where the animals were sacrificed after 31 days, most rats in the groups receiving ascorbic acid plus sulfathiazole did not have descended testes. Similar defects were found in about one half of the animals of the control group and in the group fed 2% citric acid. The animals in the hygromycin group were normal in this respect. An extensive production of intestinal gas was observed in the ascorbic acid plus sulfathiazole group as well as in the hygro-

mycin group. Much less gas was noted in the controls and in the sulfathiazole groups.

On macroscopical examination, most of the adrenal glands seemed to be normal, light brown in color, with the exception of two dark brown glands found in the group fed ascorbic acid plus sulfathiazole. The fat tissue in which the adrenals are normally imbedded was found only in the ascorbic acid and hygromycin groups. Few cases of lung infection and lung tumors were detected; however, no relation to the diet fed was apparent.

In the first series where the animals were sacrificed after 52 instead of 31 days, results similar to those of the second series were obtained. The adrenals were more seriously affected, however, being dark brown in color; in a few animals in the ascorbic acid plus sulfathiazole and the hygromycin groups they were markedly hypertrophied and hemorrhagic.

Histological examination of the adrenal glands of animals in the ascorbic acid plus sulfathiazole group revealed excessive capillary congestion of the zona reticularis; in a few cases necrotic areas between the zona reticularis and fasciculata were also observed. A few instances of capillary congestion in zona reticularis were also noted in the control group and the group receiving citric acid or hygromycin. Many animals showed a marked degeneration or loss of interstitial tissue in the testes. Again, as in the second series, the group on ascorbic acid plus sulfathiazole was most seriously affected. However, about one half of the animals in the other groups also showed this condition. Similarly, spermiogenesis was affected in all groups and this defect did not seem to be related to any special diet.

The relative weight of the adrenal glands e.g. the weight of glands per 100 gm body weight, is sometimes used as a criterion of the degree of pantothenic acid deficiency. In our experiment, the highest relative weight of adrenals was found in the ascorbic acid plus sulfathiazole and the sulfathiazole groups, namely, 50.7 and 45.6 mg respectively. The hygromycin group had the lowest average value, 26.2 mg. There was

TABLE 1

Effect of various supplements on the weight gains of pantothenic acid-deficient rats

SERIES	DAYS	PA-DEFICIENT CONTROLS	ASCORBIC ACID	SULFATHIAZOLE	AC + ST [1]	HYGROMYCIN	CITRIC ACID
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
I	1 – 10	27 ± 4.1[2]	34 ± 2.9	25 ± 2.1	24 ± 3.4	41 ± 2.0[3]	
	11 – 31	9 ± 3.5	31 ± 9.1[3]	11 ± 2.5	8 ± 3.6	20 ± 3.3[3]	
	32 – 52	23	61	0.4	——	13	
II	1 – 10	26 ± 3.0	26 ± 3.2	25 ± 1.6	22 ± 2.2	31 ± 2.2	27 ± 2.1
	11 – 31	17 ± 3.4	36 ± 7.7[3]	0.7 ± 2.7[3]	3 ± 2.0[3]	41 ± 3.5[3]	16 ± 3.5

[1] Ascorbic acid and sulfathiazole.
[2] Mean ± standard error of the mean.
[3] Significant at 0.1% level.

TABLE 2

Effects of supplements to a pantothenic acid-deficient diet on serum protein distribution

SUPPLEMENT	NO. OF SAMPLES	ALBUMIN	GLOBULINS			
			α_1	α_2	β	γ
		%	%	%	%	%
None						
Pantothenic acid-deficient control	8	36 ± 6.2[1]	19 ± 3.8	10 ± 1.6	23 ± 4.6	12 ± 3.3
Citric acid	6	48 ± 8.3[2]	17 ± 3.8	8 ± 3.4	19 ± 3.1	8 ± 2.4
Sulfathiazole	5	46 ± 2.7[2]	20 ± 2.1	9 ± 1.8	20 ± 4.0	5 ± 1.3[2]
Ascorbic acid	7	33 ± 6.7	19 ± 2.3	12 ± 3.0	19 ± 1.9	17 ± 4.9[3]
Ascorbic acid and sulfathiazole	4	38 ± 5.9	18 ± 5.3	12 ± 0.6	19 ± 4.0	13 ± 3.6
Hygromycin	8	52 ± 2.8[2]	18 ± 2.6	8 ± 2.3	14 ± 2.8[2]	8 ± 1.4[3]

[1] Mean ± standard error of the mean.
[2] Significant at the 0.1% level.
[3] Significant at the 0.5% level.

practically no difference between the relative weights of the adrenals in the other three groups, the values being 31.4, 30.0, and 29.2 mg for the control, citric acid and ascorbic acid groups respectively.

The results of the electrophoretic study are summarized in table 2. The highest average value for serum albumin was found in the hygromycin group, namely 52%. The lowest value, 33%, was seen in the ascorbic acid group. The differences are statistically significant ($P > 0.01$). The percentages of α_1-, α_2-, and β-globulins did not vary markedly from one group to another, staying in the range from 17 to 20% for α_1-, 8 to 12% for α_2-, and 19 to 23% for the β-globulins. An exception was the hygromycin group which had the rather low β-globulin value of 14%. The differences in the γ-globulin percentages were more pronounced. The sulfathiazole group showed the lowest value, 5%, and the ascorbic acid groups the highest, 17%. The differences were again statistically significant ($P > 0.01$).

DISCUSSION

In confirmation of Daft's ('51) findings, the inclusion of 2% ascorbic acid in the PA-deficient diet prevented the PA-deficiency syndrome in most cases. When fed simultaneously with sulfathiazole, however, the ascorbic acid failed to show such a beneficial effect. As sulfathiazole is believed to inhibit the activity of intestinal microorganisms (Wright and Welch, '44) it can be concluded that the favorable influence of ascorbic acid is a secondary one, mediated through changes in the intestinal microflora. This suggestion is supported by the findings that glucuronolactone, which when fed in 2% concentration in a PA-deficient diet also prevents the deficiency signs, is ineffective when given parenterally (Hundley and Ing, '53). In agreement with reports of Lih and Baumann ('51), Sauberlich ('52) and Guggenheim et al. ('54) the antibiotic agent prevented the PA-deficiency syndrome. This protection, however, was only temporary, and after about 45 days of experiment the signs of deficiency appeared.

The prevention of deficiency signs, noted in rats receiving 2% ascorbic acid seemed to be a lasting one, (Daft, '51), as compared with the only temporary protection offered by hygromycin. This observation, considered along with the differences in pathology of some vital organs and in serum protein distribution, suggests that even if these two substances seem to alleviate the signs of PA deficiency by changes in the intestinal microflora, the underlying mechanisms are probably not identical.

Pantothenic acid deficiency has been reported to lower the resistance of animals to infections. The primary cause of death in many cases is a lung infection (Zucker et al., '54; Seronde, '54). Axelrod and Pruzansky ('54) reported a decreased production of antibodies in PA-deficient rats. This finding could, at least partially, explain the lower resistance to the infections.

Although antibodies are believed to belong mainly to the γ-globulin type of proteins, Axelrod and Pruzansky ('54) did not find any difference between the vitamin-supplemented controls and the deficient animals with respect to serum γ-globulin levels. In our experiments, the variation between the individual groups with respect to the γ-globulin concentration was considerable. As the principal effects of the administered substances are believed to be some qualitative or quantitative changes (or both) in microflora, the results of our experiments suggest possible connections between the microbiological conditions of the intestinal tract and the processes regulating the synthesis of blood proteins, especially the γ-globulins.

SUMMARY

The influence of ascorbic acid, phthalylsulfathiazole, ascorbic acid and sulfathiazole combined, citric acid, and an antibiotic, hygromycin, on the physiological effects of a pantothenic acid-deficient diet were investigated in experiments with male weanling albino rats. The concentration of hygromycin was 100 or 200 mg per kilogram of diet; each of the other test substances was used at a 2% level.

The presence of either ascorbic acid or hygromycin in the pantothenic acid-deficient diet delayed or suppressed the appearance of deficiency signs. Sulfathiazole, fed simultaneously with ascorbic acid, counteracted the beneficial effect of the ascorbic acid. The addition of citric acid did not seem to influence significantly the effect of the basal deficient diet. The histological picture of the adrenals and testes, as well as the distribution of blood serum proteins, were affected markedly by the supplements. It is concluded that the favorable effect of ascorbic acid is probably a secondary one, mediated through changes in the intestinal microflora, and that these changes may not be identical with those observed when feeding hygromycin.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R. J. Floody, Hoffmann-La-Roche, Inc. for the generous supply of vitamins and Dr. R. D. Coghill, Abbott Laboratories, Inc. for the halibut liver oil. We are indebted also to Dr. A. D. Whedon for the histological examination of tissues, and to Miss Charleen Sibley for technical assistance.

LITERATURE CITED

AXELROD, A. E., AND J. PRUZANSKY 1954 Role of Vitamins in Antibody Production. Nutrition Symp. Ser. 8; National Vitamin Foundation, Inc.

DAFT, F. S. 1951 Effect of vitamin C on pantothenic acid-deficient rats. Federation Proc., *10:* 380.

DAFT, F. S., AND K. SCHWARZ 1952 Prevention of certain B-vitamin deficiencies with ascorbic acid or antibiotics. Ibid., *11:* 200.

EVERSON, G., L. NORTHROP, N. Y. CHUNG AND R. GETTY 1954 Effect of ascorbic acid on rats deprived of pantothenic acid during pregnancy. J. Nutrition, *54:* 305.

GUGGENHEIM, K., S. HALEVY, I. HARTMANN, AND R. ZAMIR 1954 The effect of antibiotics on the metabolism of certain B-vitamins. Ibid., *50:* 245.

HEGSTED, D. M., R. C. MILLS, C. A. ELVEHJEM AND E. B. HART 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.

HUNDLEY, J. M., AND R. B. ING 1953 Prevention of pantothenic acid deficiency with glucuronolactone and other compounds related to ascorbic acid. Federation Proc., *12:* 417.

JOHANSSON, K. R., G. E. PETERSON AND E. C. DICK 1953 Effect of dietary aureomycin upon intestinal microflora and the intestinal synthesis of vitamin B_{12} in the rat. J. Nutrition, *49:* 135.

LIH, H., AND C. A. BAUMANN 1951 Effect of certain antibiotics on the growth of rats fed diets limiting in thiamine, riboflavine, or pantothenic acid. Ibid., *45:* 143.

MANN, R. L., R. M. GALE AND F. R. VANABELE 1953 Hygromycin. II. Isolation and properties. Antibiotics Ann. 1953-54: 167. Med. Encyclop., Inc., New York, N. Y.

PITTENGER, R. C., R. N. WOLFE, M. M. HOEHN, P. N. MARKS, W. A. DAILY AND J. M. MCGUIRE 1953 Hygromycin. I. Preliminary studies on the production and biological activity of a new antibiotic. Antibiotics Ann. 1953-54: 157, Med. Encyclop., Inc., New York, N. Y.

SAUBERLICH, H. E. 1952 Effect of aureomycin and penicillin upon the vitamin requirement of the rat. J. Nutrition, *46:* 99.

SERONDE, J. JR. 1954 Resistance of rats to inoculation with corynebacterium pathogenic in pantothenic acid deficiency. Proc. Soc. Exp. Biol. Med., *85:* 521.

WRIGHT, L. D., AND A. D. WELCH 1944 Folic acid, biotin and pantothenic acid deficiency and the liver storage of various vitamins in rats fed succinylsulfathiazole in highly purified rations. J. Nutrition, *27:* 55.

ZUCKER, T. F., AND L. M. ZUCKER 1944 Pantothenic acid deficiency and loss of natural resistance to a bacterial infection in the rat. Proc. Soc. Exp. Biol. Med., *85:* 517.

NUTRITION STUDIES IN THE COLD

I. INFLUENCE OF DIET AND LOW ENVIRONMENTAL TEMPERATURE ON GROWTH AND ON THE LIPID CONTENT OF LIVERS IN THE RAT [1]

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(Received for publication July 19, 1957)

The response of animals maintained at low environmental temperatures to variations in diet composition has received considerable attention in recent years. There is an increase in the calorie requirement (Treichler and Mitchell, '41) during cold exposure due, at least in part, to an increase in heat production. The "resting" metabolism (Benedict and MacLeod, '29) and the overall metabolic rate (Sellers and You, '50) are elevated during exposure. The animal's response to cold appears to be mediated through the pituitary (Tyslowitz and Astwood, '42), the adrenals (Sellers, You and Thomas, '51) and the thyroid (LeBlond and Gross, '43). Associated with the increased energy requirement of animals exposed to cold is an increased requirement for specific dietary constituents. These include ascorbic acid (Dugal and Therien, '47), pyridoxine (Ershoff, '51), riboflavin (Ershoff, '52a) and vitamin A (Ershoff, '52b).

The evidence regarding the optimum proportions of the three organic foodstuffs for adjustment, survival and growth in a cold environment is somewhat conflicting and difficult of interpretation. The literature up to 1950 has been reviewed

[1] This research was supported in part by the United States Air Force under Contract no. AF 18 (600)–463, monitored by the Alaskan Air Command, Arctic Aeromedical Laboratory, APO 731, Seattle, Washington.

by Mitchell and Edman ('51) who concluded that high protein intake is not beneficial in reference to cold tolerance; that a high carbohydrate diet is superior in this respect to one high in protein; and that dietary fat under some conditions improves still further the beneficial effects of carbohydrate foods (see also Page and Babineau, '53). Sellers et al. ('54) have reported observations which indicate that variations in the fat content of the diet do not have any effect on growth rate or survival in the cold, and that the calorie intake is remarkably constant regardless of the amount of lipid in the diet. Data of Page and Babineau ('53) suggest that high-fat diets may be more efficiently utilized in the cold than low-fat ones. This possibility may be related to the finding of Kayser ('37) that fats are utilized preferentially for heat production in the cold. Young rats fed ad libitum in a cold environment exhibit a lower rate of weight increase and greater food intake than comparable animals at room temperature. Apparently the requirements for growth and the increased heat production exceed the capacity of the animal either to ingest dietary bulk or to metabolize the foodstuffs. The situation with respect to adult animals which have reached a weight plateau is not clear from the available data. From such general considerations it has seemed possible that alterations in the proportions of the foodstuffs in the diet or increased amounts of specific substances or both might benefit an animal living in a cold environment. Increasing the fat content of the diet has attractive possibilities because of the high calorie content and numerous reports that peoples in cold climates voluntarily ingest large quantities of fat and may exhibit so-called "fat hunger." However, diets unbalanced in respect to fat may lead to ketosis and its sequelae.

An extensive study of the responses of animals in a cold environment to alterations in diet composition is in progress in this laboratory. Particular emphasis has been placed on the possibility that lipotropic factors may increase or improve fat utilization. This report presents data on the growth rate, food and calorie intake, protein efficiency ratio, liver weight,

and total liver lipids of immature and young adult male rats receiving 8 different synthetic diets at room temperature and in the cold. While onr preliminary experiments were in progress, Sellers and You ('52) reported that when rats were exposed to a cold environment, the deposition of excess fat in the liver produced by feeding a hypolipotropic diet was effectively prevented. Our data confirm and extend this observation.

TABLE 1

Percentage composition of diets

CONSTITUENTS	DIETS							
	1	2	3	4	5	6	7	8
	%	%	%	%	%	%	%	%
Casein [1]	5	10	20	30	40	20	20	20
Salt mixture [2]	5	5	5	5	5	5	5	5
Cellu flour [3]	2	2	2	2	2	2	2	2
Starch	33	31	26	21	16	31	21	16
Sucrose	33	30	25	20	15	30	20	15
Lard	18	18	18	18	18	8	28	38
Vitamin mixture [4]	2	2	2	2	2	2	2	2
Cod liver oil	2	2	2	2	2	2	2	2

[1] Vitamin-free casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Hubbell, Mendel and Wakeman ('37).

[3] Purified cellulose, Chicago Dietetic Supply House, Chicago, Ill.

[4] The vitamin mixture had the following composition per kilogram: thiamine hydrochloride, 250 mg; riboflavin, 500 mg; niacin, 1000 mg; pyridoxine hydrochloride, 250 mg; calcium pantothenate, 1250 mg; biotin, 5 mg; pteroylglutamic acid, 10 mg; para-aminobenzoic acid, 6.25 gm; 2-methyl-napthoquinone, 100 mg; and sucrose, 990.4 gm.

EXPERIMENTAL

The diets employed are shown in table 1. In diets 1 to 5 the protein content was varied from 5 to 40% at the expense of carbohydrate with the fat constant at 20%. In diets 6, 3, 7, and 8 the fat was varied from 10 to 40% at the expense of carbohydrate with protein constant at 20%. Choline and inositol were omitted from the vitamin mixture [2] so that the lipotropic

[2] Unpublished experiments in this laboratory have shown that omission of vitamin E from the mixture does not influence any of the data obtained under the present experimental conditions.

factors of the diets were supplied entirely by the methionine of the dietary casein. The diets were prepared at weekly intervals and stored at 1°C.

White male rats of the Carworth strain were used in all experiments. The rats on arrival at the laboratory were placed in stock cages and received a commerical rabbit chow [3] and water, ad libitum, for 5 to 7 days. They were then weighed and assigned to various dietary groups and to the 25 ± 1° or 1 ± 1°C temperatures in a random manner. In experiment 1 the rats ranged in body weight from 60 to 80 gm; in experiment 2, from 150 to 180 gm. All animals received the diets and distilled water ad libitum. The daily food intake was determined and the animals were weighed three times per week. All of the experiments were of 28 days' duration. At the end of the experiments the animals were sacrificed and the livers removed, weighed, and analyzed for total lipids essentially as described by Dury and Treadwell ('53). During the experiments the rats were housed in individual cages with wire mesh bottoms so that coprophagy was eliminated. In a preliminary experiment at 1° with rats of 50 to 60 gm body weight, the death rate at 1° was 70 to 100% for different dietary groups. The animals which died developed a condition comparable to "frost bite" of the feet, tail, and scrotum, with edema, necrosis, and hemorrhage of these parts shortly before death. Much of the earlier work on animals in the cold had been with adult animals so the experiment was repeated with older animals (150 to 180 gm). Again the death rate (at 1°) was very high (80 to 100%) on all diets. It seemed possible that the metal cages were conducting a considerable amount of heat away from the animals so that the heat-producing mechanisms were under a much greater stress than if the animals were in contact with a non-conductor. The bottoms of the cages were then covered with a perforated plastic matting. This produced a satisfactory survival rate (see table 2) and was employed in all later experiments. The

[3] Purina.

DIET NO.	1		2	
TEMPERATURE	1°	25°	1°	25°
Number of rats	8	7	8	7
Survival, %	75	100	87	100
Food, *gm/day* [1]	11.9 ±1.0	11.6 ±1.5	14.2 ±0.5	11.9 ±1.4
Calories/day	55.2 ±4.8	53.8 ±7.2	65.9 ±2.2	55.2 ±5.6
Liver, *gm/100 gm B.W.*	5.0 ±0.1	6.5 ±0.3	4.3 ±0.1	6.1 ±0.6
Number of rats	8	6	8	6
Survival, %	87	100	100	100
Food, *gm/day*	16.7 ±0.6	15.1 ±0.9	17.1 ±0.6	13.9 ±0.6
Calories/day	77.5 ±2.9	70.1 ±4.4	82.1 ±2.6	64.5 ±3.1
Liver, *gm/100 gm B.W.*	3.8 ±0.1	4.5 ±0.2	3.7 ±0.2	3.8 ±0.2

TABLE 2

Survival, food and calorie intake at 1° and 25°C.

3		4		5		6		7		8	
1°	25°	1°	25°	1°	25°	1°	25°	1°	25°	1°	25°
Experiment I, Young Males											
8	7	8	7	8	7	8	7	8	7	10	7
100	100	100	100	100	100	100	83	75	100	60	100
15.2 ±0.5	10.1 ±0.8	13.8 ±0.4	9.3 ±1.1	14.4 ±0.6	9.3 ±0.7	16.5 ±0.9	10.6 ±0.2	13.2 ±0.2	9.6 ±0.5	11.9 ±0.9	7.5 ±0.6
70.5 ±2.5	46.9 ±3.2	64.0 ±1.9	43.2 ±5.3	66.8 ±3.0	43.2 ±3.6	64.2 ±3.4	43.9 ±0.9	67.8 ±1.3	49.3 ±2.4	67.1 ±5.1	42.3 ±3.4
4.7 ±0.1	5.7 ±0.4	4.5 ±0.1	4.7 ±0.2	4.8 ±0.1	4.2 ±0.1	4.4 ±0.1	4.2 ±0.4	4.4 ±0.2	6.2 ±0.4	4.4 ±0.1	6.5 ±0.4
Experiment II, Adult Males											
8	6	8	6	8	6	8	6	8	6	8	6
100	100	87	100	100	100	100	100	100	100	100	100
16.0 ±0.3	14.3 ±0.4	17.5 ±0.6	13.5 ±0.2	16.9 ±0.5	13.5 ±0.4	20.5 ±1.0	12.5 ±0.4	16.3 ±0.4	12.6 ±0.4	15.4 ±0.6	12.2 ±0.6
74.2 ±1.6	66.4 ±1.9	81.2 ±2.8	62.6 ±1.1	78.4 ±2.2	62.6 ±1.9	82.8 ±4.2	51.8 ±1.9	83.8 ±2.1	64.8 ±2.0	86.9 ±3.7	68.8 ±3.0
3.7 ±0.1	3.7 ±0.1	3.7 ±0.1	3.5 ±0.4	3.8 ±0.1	3.5 ±0.1	3.5 ±0.1	3.8 ±0.5	3.5 ±0.1	4.1 ±0.4	3.6 ±0.1	4.1 ±0.3

data obtained in experiments I and II are summarized in table 2 and in figures 1–3. The individual values are the means for the number of animals indicated in table 2. The liver lipids were calculated as percentage of wet weight and as grams of

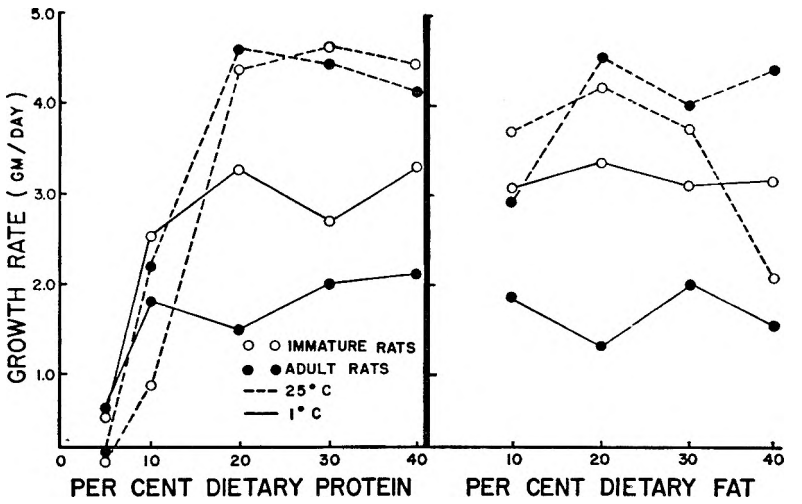

Fig. 1 Growth rate in relation to level of dietary protein and dietary fat.

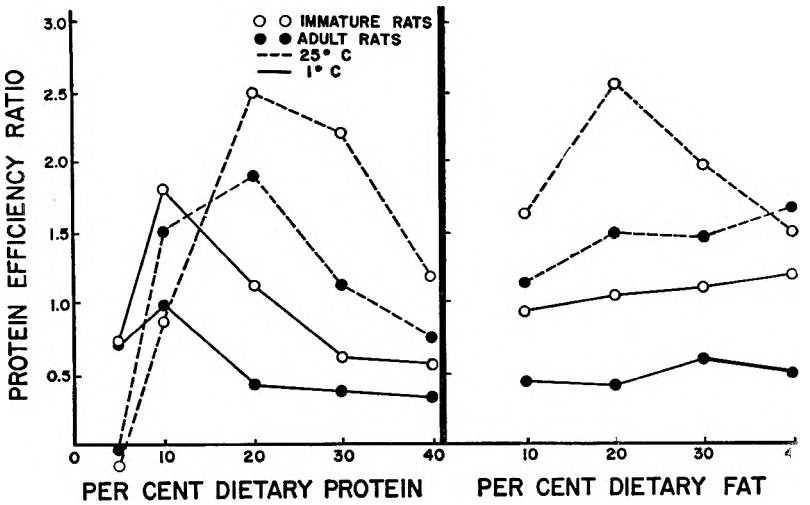

Fig. 2 Protein efficiency ratio (grams change in body weight/grams protein ingested) in relation to level of dietary protein and dietary fat.

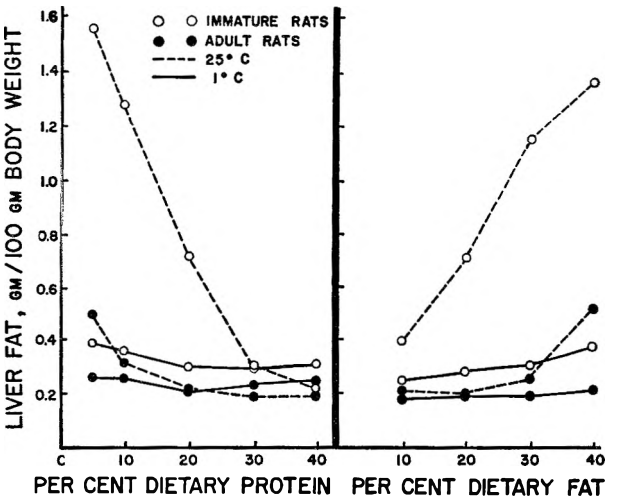

Fig. 3 Liver fat in relation to level of dietary protein and dietary fat.

total lipids per 100 gm body weight. These two modes of calculation led to the same general conclusions so only the latter is shown in the figures. Apparent differences were analyzed for significance by the *t* method of Fisher ('38) and only those showing a P value less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Experiment I, immature male rats, 60 to 80 gm initial weight

At 25° the survival was 100% for all dietary groups except no. 6 in which one animal died due to a respiratory infection. The data at 1° suggest that there was a decreased survival at low protein levels (5 and 10%) and at high fat levels. However, the number of animals is too limited to allow valid conclusions. The food and calorie intake was higher at 1° than at 25° on all diets except no. 1 which contained 5% protein. The finding for diet 1 is particularly interesting in relation to the growth rate and protein efficiency ratio, as will be discussed below. At 1° on diets 2 through 8 the food intake varied with calorie content but the calorie intake was essentially constant (range 64.0 to 70.5 Cal/day). This is in agreement with

the report of Sellers et al. ('54). This was also true for the rats at 25° on diets 3 through 8. Thus it appears that if there is sufficient protein in the diet to support minimal growth at a particular environmental temperature then the calorie intake at that temperature will be constant regardless of the calorie content. The livers of the animals at 25° were larger on the low-protein and the high-fat diets, (no. 1, 2, 7, 8) than those of the rats at 1°. The liver lipid data indicate that these differences were due to the relatively hypolipotropic nature of these diets at 25°.

The relationship of the protein content of the diet to the growth rate is shown in figure 1. At the 5 and 10% protein levels the growth rate was distinctly greater at 1° than at 25°. With 5% protein the food and calorie intake was essentially the same at both temperatures so that the greater growth rate at 1° was apparently due to a more efficient utilization of the available protein (fig. 2). The increased growth at 1° with 10% protein was due to more efficient utilization (fig. 2) and to a greater supply of protein because of the greater food intake. The situation was reversed at protein levels above 10%. The animals at 25° had a higher growth rate and utilized the available protein more efficiently for growth. The comparable animals at 1° had a higher protein and calorie intake. The most probable explanation is that when the protein level was increased the carbohydrate content was decreased and with a constant calorie intake the amount of protein being utilized for energy at 1° was increasing so that no more protein was available for growth than at the 10% level. The maximum growth rate at 1° was obtained with the 10% protein level and at 25°, with the 20% level.

When the protein content was held constant at 20% and the fat content varied from 10 to 40% the growth rate at 25° was higher at the 10, 20, and 30% fat levels and lower at the 40% than at 1° (fig. 1). The protein efficiency ratio at 1° gradually increased with increasing fat content so that with 40% fat there was essentially no difference between the two temperatures (fig. 2). Figure 1 shows that the better growth on 40%

fat at 1° was due to a depression of the rate at 25° rather than an increase in rate at 1°. Higher levels of fat and other levels of protein should be investigated in this respect.

Figure 3 shows the liver lipid levels in the immature rats in relation to the protein and fat content of the diets at the two temperatures. It will be noted that at 25° the liver lipids were highest on the 5% protein diet and dropped regularly and sharply with increased protein levels up to 30%. The further slight decrease at 40% protein is of questionable significance. The very interesting finding is that the liver lipid level at 1° was essentially the same at all protein levels and in the normal range for the rat. Increasing the fat content from 10 to 40% produced a sharp and regular increase in liver lipids at 25°, while at 1° there was no significant change, and at all fat levels the liver lipids were in the normal range. These findings clearly show that cold is an effective lipotropic agent. It will be recalled that these diets were free of choline and inositol and that the only recognized lipotropic agent present was the methionine of the dietary casein. The lipotropic agents are generally believed to act by promoting the formation of phospholipids in the liver and thereby promoting fat utilization; in their absence, neutral fat accumulates in the liver because of the failure in phospholipid formation. In these experiments at 1° it seems certain that fat was being utilized at a rapid rate in the absence of an adequate dietary supply of lipotropic factors. It would appear that the animals at 1° (a) were able to utilize fat through some process which does not involve lipotropic factors, (2) used the available supply of lipotropic factors more efficiently, or (3) produced a supply of lipotropic factors from endogenous sources (see also Sellers and You, '52). One possible endogenous source would be from increased catabolism of protein with the liberation of methyl groups and this may be a factor in some of the dietary groups where the protein intake was greater, the protein efficiency ratio less, and the growth rate lower at 1° than at 25°. However with diets 1 and 2 this does not appear likely. On diet 1 the animals ingested practically the same

amount of food at both temperatures yet at 1° the growth rate and protein efficiency ratio was higher and the liver fat was normal. It is obvious from the data that, using the level of liver lipids as a criterion, the rat at 1° has an increased capacity for metabolizing fat.

Experiment II, young adult male rats, 150 to 180 gm initial weight

Qualitatively, the adult rats exhibited the same differences at the two temperatures in relation to the composition of the diets as was found for the immature rats (figs. 1–3). At 10% protein the adult animals had a smaller growth rate difference between the two temperatures than the immature rats while above this level the difference was greater for the adults. This was due to a difference in growth rate between the young and adult animals at 1°. At 1° the growth rate was constant for all fat levels (20% protein); at 25° the growth rate tended to increase with elevation in fat.

It was reported by Horning and Eckstein ('44) that adult rats need less methionine for increases in weight and hence more is available for lipotropic action than in young rats. In the present experiment the adult rats at 25° had a significant elevation in liver fat on diets 1 and 8 while the young rats had above normal liver lipids on diets 1, 2, 3, 7, and 8. The liver lipids were in the normal range in the adults on diets 1 and 8 at 1°. Thus, cold had a lipotropic effect in the adult rat also.

An interesting general aspect of these two experiments is that while with other essential dietary constituents stresses such as cold, heat, etc. increase the requirements (Ershoff, '52, a, b) cold stress appears to decrease the dietary requirement for lipotropic factors.

SUMMARY

Immature and young adult male rats received a series of 8 choline-and inositol-free diets varying in protein content from 5 to 40% (with 20% fat) and in fat from 10 to 40% (with 20% protein). Parallel groups were maintained at 1° and at 25°C.

With the immature rats at 5 and 10% protein levels the growth rate and protein efficiency ratio were higher at 1° than at 25°. The reverse was true at protein levels of 20, 30 and 40%. The growth rate was higher at 25° than at 1° on the 10, 20, and 30% fat levels. With 40% fat the growth rate was higher at 1°.

At 25° the liver lipids in the immature rats were highest on the 5% protein diet and dropped regularly and sharply up to 30% protein. At 1° the liver lipids were essentially the same at all protein levels and were in the normal range for the rat. Increasing the fat content of the diet from 10 to 40% produced a regular increase in liver lipids at 25° while at 1° there was no significant change and the liver lipids were in the normal range at all fat levels.

The observations on the young adult animals were qualitatively the same as for the immature rats. The dietary requirement for lipotropic factors appeared to be lower at 25° for the adult animals.

The food and calorie intake was higher at 1° for both groups of animals. The mechanism of the lipotropic effect of cold is discussed.

LITERATURE CITED

BENEDICT, F. G., AND G. MACLEOD 1929 Heat production of albino rat; influence of environmental temperature, age and sex; Comparison with basal metabolism of man. J. Nutrition, *1:* 367.

DUGAL, L. P., AND M. THERIEN 1947 Ascorbic acid and acclimatization to cold environment. Canad. J. Research., *25E:* 111.

DURY, A., AND C. R. TREADWELL 1953 Hormonal influences on liver lipid partition, carbohydrate and electrolyte metabolism in the rat. Proc. Soc. Exp. Biol. Med., *82:* 719.

ERSHOFF, B. H. 1951 Decreased resistance of pyridoxine-deficient rats to cold exposure. Ibid., *78:* 385.

———— 1952a Decreased resistance of pyridoxine-deficient rats to cold stress. Ibid., *79:* 559.

———— 1952b Effects of vitamin A malnutrition on resistance to stress. Ibid., *79:* 580.

FISHER, R. A. 1938 Statistical Methods for Research Workers, 7th Ed., Oliver and Boyd, Edinburgh.

HORNING, M. G., AND H. C. ECKSTEIN 1944 The influence of supplementary casein cystine and methionine on liver lipid content of adult rats. J. Biol. Chem., *155:* 49.

HUBBELL, R. B., L. B. MENDEL AND A. J. WAKEMAN 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14:* 273.

KAYSER, C. 1937 Les lipides assurent preferentiellement la thermogenese de rechauffement chez le cobaye. Compt. Rend. Soc. Biol., *126:* 701.

LEBLOND, C. P., AND J. GROSS 1943 Effect of thyroidectomy on resistance to low environmental temperature. Endocrinology, *33:* 155.

MITCHELL, H. H., AND M. EDMAN 1951 Nutrition and Climatic Stress, with Particular Reference to Man. Charles C. Thomas Publishing Co., Springfield, Ill.

PAGE, E., AND L. BABINEAU 1953 The effects of diet and cold on body composition and fat distribution in the white rat. Canad. J. Med. Sci., *31:* 22.

SELLERS, E. A., AND S. S. YOU 1950 Role of the thyroid in metabolic responses to a cold environment. Am. J. Physiol., *163:* 81.

———— 1952 Effects of cold environment on deposition of fat in the liver in choline deficiency. Biochem. J., *51:* 573.

SELLERS, E. A., S. S. YOU AND N. THOMAS 1951 Acclimatization and survival of rats in the cold: effects of clipping, of adrenalectomy and of Thyroidectomy. Am. J. Physiol., *165:* 481.

SELLERS, E. A., R. W. YOU AND N. M. MOFFAT 1954 Regulation of food consumption by caloric value of the ration in rats exposed to cold. Ibid., *177:* 367.

TREICHLER, R., AND H. H. MITCHELL 1941 Influence of plane of nutrition and of environmental temperature on relationship between basal metabolism and endogenous nitrogen metabolism subsequently determined. J. Nutrition, *22:* 333.

TYSLOWITZ, R., AND E. B. ARTWOOD 1942 Influence of pituitary and adrenal cortex on resistance to low environmental temperature. Am. J. Physiol., *136:* 22.

PYRIDOXAL-5-PHOSPHORIC ACID IN WHOLE BLOOD AND ISOLATED LEUKOCYTES OF MAN AND ANIMALS

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(Received for publication July 12, 1957)

Investigations on the state of vitamin B_6 nutrition in man have been concerned with the determination of dietary intake, absorption and urinary excretion of the vitamin and its oxidation products (Rabinowitz and Snell, '49). An estimate of its functional sufficiency has been obtained by measurements of xanthurenic acid and N-methylnicotinamide excretion after tryptophan load tests (Sarett and Goldsmith, '49). The occurrence of vitamin B_6 deficiency in infants has been demonstrated by Molony and Parmelee ('54) and Coursin ('54), and the frequent occurrence of inadequate vitamin B_6 intake to meet increased requirements during pregnancy has been inferred by Wachstein and Guadaitis ('53) from results of tryptophan load tests. Vilter et al. ('53) have studied the symptomology of vitamin B_6 deficiency in the adult with the aid of the vitamin antagonist desoxypyridoxine.

It is in the area of amino acid metabolism that the most detailed biochemical insight into the function of vitamin B_6 is available (Fried and Lardy, '55). The coenzyme forms of the vitamin, pyridoxal phosphoric acid (B_6-PO_4) and to some extent pyridoxamine phosphoric acid, serve as catalysts in a wide variety of biochemical reactions of amino acids. Pyridoxal phosphate is primarily an intracellular constituent and

has been measured in a variety of animal tissues (Umbreit et al., '45). Measurements of this coenzyme in the whole blood and isolated leukocytes of man and some animals are reported here.

EXPERIMENTAL

Pyridoxal phosphate in whole blood was determined by a modification of the manometric method of Umbreit et al. ('45). The method is based on the measurement of the rate of CO_2 development from the decarboxylation of tyrosine by a B_6-PO_4-activated tyrosine decarboxylase. A suspension of freeze-dried cells of *Streptococcus faecalis R,* (no. 8043) serves as the source of a nearly B_6-PO_4-free apoenzyme.

The sensitivity of the method was increased 4- to 5-fold by mechanical changes in the manometric measurement and the precision was improved by rearrangement of the assay set up. Single side-arm Warburg vessels of a total volume of about 5 ml [1] were used and the manometer fluid was replaced by n-heptane (Sp. Gr. 0.71) stained with Sudan III. These mechanical changes in the manometry permitted a 4- to 5-fold increase in sensitivity but in order to maintain the desired precision, the water bath had to be controlled to $\pm$ 0.005°C. and certain rearrangements in the assay system had to be made.

Determination of B_6-PO_4 in whole blood. Blood was either oxalated or immediately frozen in dry ice. The blood, protected from light, could be kept at refrigerator temperature for 24 hours or in dry ice 72 hours without measurable loss of B_6-PO_4. The sample was hemolyzed with 2 vol. of distilled water and used directly for assay. Heating with alkali, necessary for liberation of B_6-PO_4 from tissue, was found unnecessary in blood, all B_6-PO_4 being either free or readily available to the apoenzyme. The blood had to be diluted with at least 2 vol. of water, since undiluted blood or 1:1 diluted blood slightly inhibited the analytical recovery of added B_6-PO_4.

To the main compartment of the Warburg flask was added 0.2 ml M/5 acetate buffer pH 5.5, 0.1 ml of a suspension of

[1] American Instrument Co., no. 5–203.

excess tryosine in buffer (approximately 50 mg/ml, exact concentration irrelevant), 0.3 ml H_2O and 0.2 ml of the diluted blood or the appropriate dilution of B_6-PO_4 for a standard curve. To the side-arm was added 0.2 ml of the cell suspension. The concentration of the cell suspension varied from preparation to preparation within the range of 6 to 12 mg/ml. For a given preparation, the concentration of the suspension was so adjusted that a reading of about 30 manometer divisions was obtained in 15 minutes when 1 mμg of B_6-PO_4 was added to the flask.

After a 10-minute equilibration period, the contents of the side-arm were tipped in and three consecutive 5-minute readings were taken. The cumulative CO_2 development for the 15-minute period was used for the calculations. A calibration curve using amounts of B_6-PO_4 ranging from 0.5 to 5 mμg per flask was constructed. With a given apoenzyme preparation, the calibration curve stays quite constant from day to day. The curve starts flattening out above 4 mμg and amounts above 5 mμg cannot be estimated. Taking the dilution factor into account, the detection limit lies at 10 mμg of B_6-PO_4 per 1 ml of undiluted blood. The precision of the measurement is also about $\pm$ 10 mμg/ml.

Determination of B_6-PO_4 in leukocytes. Leukocytes were isolated from human blood by a variation of the method of Li and Osgood ('49) using freeze-dried hemagglutinin prepared from navy beans. Five to 10 ml of blood was collected in 100 $\times$ 16 mm, siliconed test tubes covered with a film of finely powdered di-sodium versenate, and closed with a polyethylene stopper. The blood, protected from light, was kept at 0 to 4°C. and the leukocytes were isolated within 24 hours.

The blood is mixed thoroughly by gentle inversion of the tube. Three drops of the freshly prepared hemagglutinin solution (1 mg/ml of saline) are added and the contents again gently mixed. The sudden start of the agglutination (within 1 to 2 minutes) is readily noticed by the curdy appearance of the blood on the side of the tube. The tube is now centrifuged

for one minute [2] at about two-thirds maximum speed. After centrifugation, the appearance of the tube should be as follows: at the bottom, a solid layer of agglutinated erythrocytes covered by a buffy coat of leukocytes and a supernatant layer of somewhat turbid plasma. By gentle rotary swirling, the buffy coat and some of the erythrocytes are redistributed

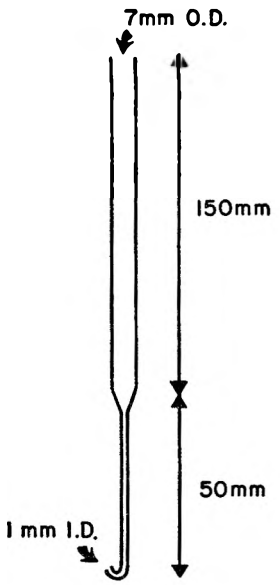

FIG. 1

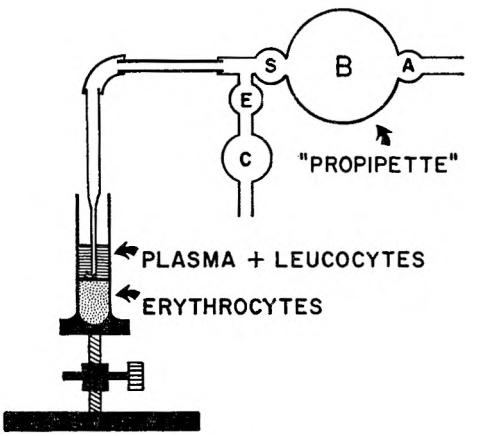

FIG. 2

Figs. 1 and 2 Apparatus for separation of leukocytes from agglutinated blood.

throughout the plasma. The tube is now centrifuged for 30 seconds at the lowest possible speed of the centrifuge. After this centrifugation, a solid layer of agglutinated erythrocytes should be covered by a layer of turbid plasma, but *no* buffy coat should be apparent.

[2] International Clinical Desk Centrifuge with a 4-place swinging-cup head for 15-ml tubes.

The plasma containing the suspended leukocytes is now carefully withdrawn from the packed erythrocytes using a device similar to the one of figure 1. The bulb B of a "Propipette" [3] is evacuated by squeezing it and vent A. By gentle pressure on vent S, the plasma and suspended leukocytes are slowly and cleanly aspirated into the pipette. Release of the pressure on vent S holds the plasma securely in the pipette and the test tube can be safely withdrawn. The contents of the pipette are expelled into a graduated centrifuge tube by pressure on vent E, and finally on vent E and the closed-off bulb C. The volume is noted and the leukocytes in the carefully mixed plasma are counted. From the volume of plasma and the count of leukocytes per cubic milliliter, the total number of leukocytes can be calculated readily. The efficiency of leukocyte isolation varied from 60 to 90% of the whole blood leukocytes and repeated differential counts indicated that no measurable separation of white cells had occurred.

The graduated centrifuge tube, containing an accurately known number of leukocytes suspended in plasma is now centrifuged at top speed for 5 minutes. The plasma is poured off from the packed leukocytes and the leukocytes are resuspended in 1.5 ml of 0.3% NaCl. (Most of the adhering erythrocytes are hemolyzed at this point. Previous experiments established that even three washings with 0.3% NaCl do not remove any appreciable quantities of B_6-PO_4.) The leukocytes are again packed by centrifugation and the washing discarded. To the packed leukocytes 0.25 ml of 0.5 *N* NaOH is added, the cells are dispersed and the tube is immersed in a boiling water bath for 5 minutes with occasional shaking. The alkaline hydrolysate may be kept frozen in dry ice for as long as 48 hours prior to assay. The alkaline hydrolysate is neutralized by the addition of 0.25 ml of 0.5 *N* HCl. The precipitate is removed by centrifugation and an aliquot (0.1 to 0.4 ml) of the supernatant is used for the manometric B_6-PO_4 assay as described for whole blood.

[3] Instrumentation Associates, New York.

RESULTS

Pyridoxal phosphate levels in whole blood. Preliminary to using the method on human bloods, the pyridoxal-phosphate levels of a variety of laboratory animals on a stock diet were determined.

It is apparent that wide differences exist between species and that even within a species the levels vary appreciably. In many instances the distribution between cells and plasma was determined and about two-thirds of the B_6-PO_4 was found in the packed cells, only one-third being in the plasma. On a

TABLE 1

Pyridoxal phosphate in whole blood of animals

SPECIES	NO. OF OBSERVATIONS	AV.	± S.D.	RANGE
		mμg/ml	*mμg/ml*	*mμg/ml*
Guinea pig	6	32	± 10	23 – 45
Rat	8	187	± 30	135 – 240
Rabbit	5	118	± 53	76 – 201
Cat	10	110	± 40	72 – 209
Dog	18	49	± 15	15 – 67
Rhesus monkey	39	25 below 10 mμg/ml	14 above 10 mμg/ml	Highest value 25 mμg/ml

few samples from birds, i.e., pigeons, chicks and turkeys, very high values, above 200 mμg/ml were found, most of the B_6-PO_4 being in the erythrocytes. Higher values, approaching those of body tissues in general, would indeed be expected in the nucleated red cells of birds.

In all the common species of laboratory animals, the method was sufficiently sensitive to determine the normal B_6-PO_4 levels in the blood. In the rhesus monkey and man, however, in the majority of instances no measurable B_6-PO_4 was found in the whole blood (tables 1 and 2), hence, B_6-PO_4 is either absent or its concentration is below 10 mμg/ml, the detection limit of the method. The absence of B_6-PO_4 is not due to an inhibition of the assay system by unknown factors since added

B_6-PO_4 can be quantitatively recovered and administration of oral vitamin B_6 leads to a prompt rise in the B_6-PO_4 level in the blood of man as demonstrated in table 4.

Particular attention was paid to a possible correlation between clinically present arteriosclerosis and the observed B_6-PO_4 levels, since sclerotic lesions in the smaller arteries had been observed by Rinehart and Greenberg ('51) in monkeys on vitamin B_6-deficient diets. The hospital population

TABLE 2

Pyridoxal phosphate in the whole blood of human adults

	ROUTINE PHYSICAL CHECK UP	HOSPITAL WARD
Age range, yr.	17–62	25–75
Total no.	113	111
No. below 10 mμg/ml	102	90
No. above 10 mμg/ml	11	21
Highest value observed, mμg/ml	37	36

TABLE 3

Pyridoxal phosphate in whole blood of children and infants

	PREMATURE	0–18 mo.	5–13 yr.
No.	8	10	15
Av., *mμg/ml*	32	30	
± S.D., *mμg/ml*	± 8	± 9	
Below 10 mμg/ml	0	0	15
Above 10 mμg/ml	8	10	0
Highest mμg/ml	46	45	

contained a preponderance of elderly patients and a selected group of elderly patients with manifest arteriosclerosis. Measurable B_6-PO_4 levels, however, were found in the elderly patients about as frequently as among the younger group coming in for a routine physical checkup.

There was one highly significant correlation between age and B_6-PO_4 levels in the whole blood. As indicated in table 3, the blood of all 18 children younger than 18 months contained readily measurable levels of B_6-PO_4 averaging about 33

mμg/ml, while the children in the older age group showed the same findings as the adults. There was no difference between the B_6-PO_4 levels in premature infants and children up to 18 months of age.

The effect of increased dietary vitamin B_6 intake on the whole blood B_6-PO_4 has been measured on the same patients whose B_6-PO_4 levels, before addition of extra vitamin B_6 to

TABLE 4

Effect of oral administration of vitamin B_6 on pyridoxal phosphate concentration in whole blood

DOSE	NO. OF OBSERVATIONS	B_6-PO_4 FOUND IN BLOOD		
		AV.	± S.D.	RANGE
mg/day		*mμg/ml*	*mμg/ml*	*mμg/ml*
Pyridoxine HCl, 0.5	10	19	± 9	> 10 – 37
Pyridoxine HCl, 1	32	24	± 10	15 – 55
Pyridoxine HCl, 2.5	38	38	± 15	16 – 65
Pyridoxine HCl, 5	26	44	± 19	23 – 83
Pyridoxamine-2-HCL, 5	18	37	± 8	18 – 51
Pyridoxine HCl, 25	2			52 – 54
Pyridoxine HCl, 50	10	42	± 17	25 – 65

the diet, were recorded in tables 1 and 2. The vitamin B_6 (as crystalline pyridoxine HCl, pyridoxamine -2-HCl or in some instances pyridoxal HCl) was diluted with lactose and given daily in capsules. The blood sample was drawn 2 to 4 hours after the administration of the capsule. The values recorded in the table were reached within three days and stayed more or less constant as long as the oral vitamin B_6 capsules were continued. In each instance the vitamin was given for at least 14 days. The value recorded for each individual patient is the

average of at least three measurements obtained after equilibrium had been reached. When the vitamin was discontinued, the blood levels fell slowly, reaching the detection limit within 5 to 7 days.

The data in table 4 clearly indicate that, in the presence of a sufficiently large vitamin B_6 intake, circulating B_6-PO_4 is found in the blood of man. The ability of the body to convert oral vitamin B_6 into the coenzymatically active form, i.e., B_6-PO_4, is however, not unlimited. An intake of somewhere between 4 to 7 mg of vitamin B_6, that is, an estimated 1 to 2 mg from the natural diet plus 2.5 to 5 mg given in pure form, can be converted efficiently to B_6-PO_4. At this level the system forming B_6-PO_4 apparently is saturated and even a 10-fold increase in the oral vitamin B_6 dose does not produce a further increase in the circulating B_6-PO_4. The method used does not indicate whether the excess vitamin B_6 is absorbed and then stored or excreted in the urine. The data suggest, however, that storage is only small since, even after the large doses, the blood B_6-PO_4 reaches the pretreatment level, i.e., below 10 mμg/ml, within one week. The level of oral vitamin B_6 intake at which saturation with respect to circulating B_6-PO_4 is reached is therefore somewhere between 4 to 7 mg per day for man. It is of some interest that Vilter et al. ('53) from clinical studies of refeeding patients after desoxypyridoxine-induced deficiency arrived at nearly the same figure for the optimal vitamin B_6 intake in man.

No difference between pyridoxine and pyridoxamine absorption is indicated from the data in table 4. Pyridoxal, given to a smaller number of patients at the various doses also led to the same B_6-PO_4 blood levels. Judging from these data, no difference in the efficiency of absorption and conversion to B_6-PO_4 is apparent in man.

Pyridoxal phosphate levels in leukocytes. It was clear from the outset that B_6-PO_4 is primarily an intracellular coenzyme and that estimates of the supply of the functional form of vitamin B_6 should be really meaningful only when obtained on metabolically active tissues. Since biopsies of fixed tissues

in man, on a survey basis, generally are impractical, we turned to the isolated leukocyte as the most readily available active tissue. It was essential to use a method that would permit as rapid and simple an isolation of leukocytes as possible and, of the large number of methods available, the phytohemagglutinin method of Li and Osgood proved most useful. Other methods give essentially the same values for leukocyte B_6-PO_4 but proved less simple and reproducible in our hands. The percentage of leukocytes isolated by any procedure proved not to be sufficiently reproducible to interpret the assays directly in terms of the original volume of blood used. Instead, the leukocytes present in the sample used for assay were counted accurately and the values are reported in millimicrograms of B_6-PO_4 per million leukocytes.

B_6-PO_4 is quite readily measurable in the leukocytes isolated from 5 to 10 ml of blood and increases or decreases in the value can be determined. A rough estimate of the B_6-PO_4 concentration per gram of leukocyte can be made from the data obtained. On the average, the B_6-PO_4 concentration of normal adults was 0.15 mμg per 10^6 leukocytes. Assuming 1 ml of blood to contain about 5×10^6 leukocytes weighing 0.5 mg, it is readily calculated that the B_6-PO_4 concentration in leukocytes is of the order of 1 to 2 μg/gm of leukocytes. This is the same order of magnitude found for other tissues, e.g., 6 to 9 μg/gm for liver, 2 to 5 μg/gm for muscle and 0.5 – 2 μg/gm for nervous tissue.

In figure 3, the distribution of values for leukocyte B_6-PO_4 in 4 population groups is indicated.

The two large groups of adults were obtained from a N. Y. C. Nutrition Clinic [4] and from a Plant Health department, from employees coming in for a routine checkup. Inspection of the distribution curve indicates that the two groups are similar with respect to B_6-PO_4 content of leukocytes. (see table 5.) The one striking feature is the occurrence of 17 cases among the 229 from the Nutrition Clinic where essentially no measurable B_6-PO_4 was found in the leukocytes. There was no ob-

[4] Washington Heights Health Center, Department of Health, City of New York.

vious correlation in these 17 patients between the low leukocyte B_6-PO_4 and clinical symptoms.

The two remaining groups are children in the 11 to 13 year age group. The New York school children represent a random sample obtained in a middle income school district, while the Cuban children represent a statistical sample from a nutritional survey of Cuban children in urban and rural areas and represent preponderantly the lowest economic strata.

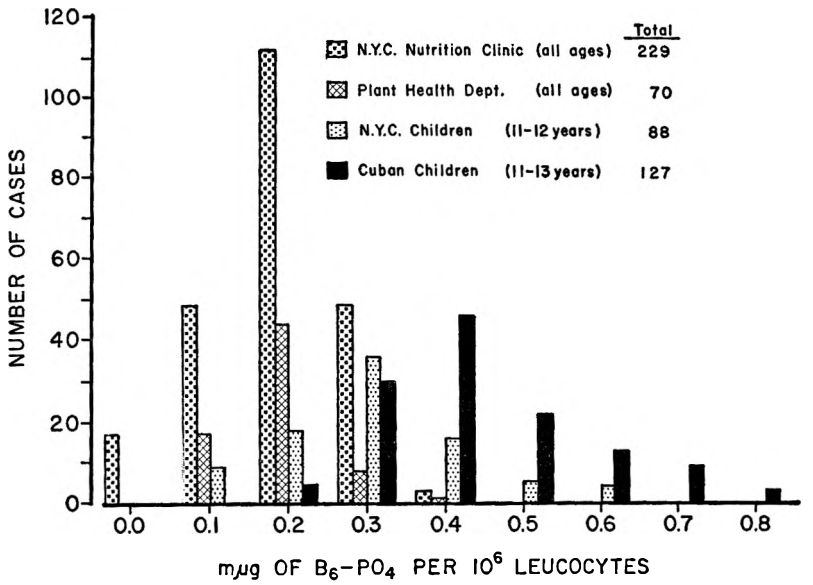

Fig. 3 Distribution of leucocyte B_6-PO_4 values among different population and age groups.

Simple inspection of the distribution data indicates that both groups of children show higher leukocyte B_6-PO_4 values than the adults and that the Cuban children in turn show higher values than the group of New York school children.

These differences are statistically highly significant as indicated by the data in table 5. The higher leukocyte B_6-PO_4 of the 11 to 12-year old children, together with the observation that in children up to 18 months even free B_6-PO_4 is found in the circulating blood, make it attractive to consider a

regression of B_6-PO_4 content, and probably demand, of tissues with age. A more extensive study of various age groups is necessary to establish this point.

The significantly higher values of the Cuban school children are more difficult to assess. First of all, comparative data on a sample of the adult population are not available and, secondly, reliable data on the vitamin B_6 content of Cuban diets or staples are not available. It is of interest to note that some of the variety of beans which form an important staple of the Cuban diet are quite high in vitamin B_6 content, approaching the values found in the best meats. The pos-

TABLE 5

Averages of B_6-PO_4 content of human leukocytes

	Average ± S.D. mμg of B_6-PO_4 per 10^9 Leukocytes	Significance of difference			
N. Y. C. Nutrition Clinic [1]	0.15 ± 0.07	> 0.50	< 0.01	< 0.01	< 0.01
Plant Health Dept.	0.14 ± 0.07				
Cuban children	0.38 ± 0.14	< 0.01			
N. Y. C. children	0.24 ± 0.12				

[1] Value excludes 17 cases in which no B_6-PO_4 was detectable.

sibility exists that less destructive methods of food preparation also might add to the available vitamin B_6 supply.

In separate experiments, it has been established that increases in vitamin B_6 intake can indeed lead to increase in leukocyte B_6-PO_4. While the B_6-PO_4 level in circulating blood rises within three days of increased oral vitamin B_6 intake (table 4), it takes 10 to 20 days before the leukocyte B_6-PO_4 responds to increased vitamin B_6 intake.

Exploratory data on the significance of leukocyte B_6-PO_4 content in arteriosclerotic disease have been obtained. In a group of 36 patients with manifest arteriosclerotic disease, there were 7 cases with barely measurable leukocyte B_6-PO_4 content (less than 0.05 mμg/10^6 leukocytes). This is a higher percentage than in the samples of the general population, but there was no obvious correlation between clinical finding and

the leukocyte B_6-PO_4 level. A larger sample and more detailed studies of the leukocyte B_6-PO_4 level is indicated and time will be required until a meaningful correlation with clinical findings can be expected.

SUMMARY AND CONCLUSIONS

Methods are described for the determination of pyridoxal-5-phosphoric acid (B_6-PO_4), codecarboxylase, in whole blood and in isolated leukocytes.

While B_6-PO_4 is measurable in the blood of most animal species investigated, levels below the detection limit, i.e., less than 10 mμg/ml are found in over 80% of adult humans. Children up to about 18 months of age show readily measurable B_6-PO_4 levels in whole blood. Oral feeding of any form of vitamin B_6 leads to a prompt rise in whole blood B_6-PO_4 in man, but a saturation level of about 60 mμg/ml is reached on feeding 4 to 7 mg of vitamin B_6.

The B_6-PO_4 content of leukocytes can be readily measured in the leukocytes isolated from 5 to 10 ml of human blood. In two large groups of adults an average of 0.15 ± 0.07 mμg of B_6-PO_4 per 10^6 leukocytes has been found. A significantly higher value was found in a group of New York school children (11 to 12 years) indicating the possibility of regression of leukocyte B_6-PO_4 with age. A sample of Cuban school children of the same age group showed even higher leukocyte B_6-PO_4 figures and the possible significance in terms of diet is discussed.

A high percentage of very low leukocyte B_6-PO_4 values was found in a small group of arteriosclerotic patients. Additional data will be required to establish correlation between this finding and clinical observations.

ACKNOWLEDGMENTS

The authors are greatly indebted to Dr. L. E. Holt, Jr., Prof. of Pediatrics, N. Y. University College of Medicine, Bellevue Hospital, N. Y., for providing samples from children under 18 months of age and to Drs. P. L. Gilbert and R. A.

Shimm, 1st (Columbia) Medical Division, Bellevue Hospital, N. Y. for samples from the hospitalized patients. The samples from persons coming in for a routine check up were obtained through the kind cooperation of Dr. S. M. Hoch, Plant Health Department of MERCK and CO., Inc., Rahway. We are indebted to the National Vitamin Foundation, The Nutrition Clinics Fund and the Williams-Waterman Fund for partial financial assistance. We also wish to thank the Fundacion de Investigaciones Medicas, Havana, Cuba, for their laboratory facilities and technical assistance.

LITERATURE CITED

COURSIN, D. B. 1954 Convulsive seizures in infants with pyridoxine deficient diet. J. Am. Med. Assoc., *154:* 406–408.

FRIED, R., AND H. LARDY 1955 Water-soluble vitamins, Part II (vitamin B_6, niacin, biotin, ascorbic acid). Ann. Rev. of Biochem., *24:* 393–400.

LI, J. G., AND E. E. OSGOOD 1949 A method for the rapid separation of leukocytes and nucleated erythrocytes from blood or marrow with a phytohemagglutinin from red beans *(Phasealus vulgaris)*. Blood, *4:* 670–675.

MOLONY, C. J., AND A. H. PARMELEE 1954 Convulsions in young infants as a result of pyridoxine (vitamin B_6) deficiency. J. Am. Med. Assoc., *154:* 405–406.

RABINOWITZ, J. C., AND E. E. SNELL 1949 Vitamin B_6 group. X V. Urinary excretion of pyridoxal, pyridoxamine, pyridoxine, and 4-pyridoxic acid in human subjects. Proc. Soc. Exp. Biol. Med., *70:* 235–240.

RINEHART, J. F., AND L. D. GREENBERG 1951 Pathogenesis of experimental arteriosclerosis in pyridoxine deficiency, with notes on similarities to human arteriosclerosis. A.M.A. Arch. Path., *51:* 12–18.

SARETT, H. P., AND G. A. GOLDSMITH 1949 Tryptophan and nicotinic acid studies in man. J. Biol. Chem., *177:* 461–475.

UMBREIT, W. W., W. D. BELLAMY AND I. C. GUNSALUS 1945 The function of pyridoxine derivatives: A comparison of natural and synthetic codecarboxylase. Arch. Biochem. Biophys., *7:* 185–199.

VILTER, R. W., J. F. MUELLER, H. S. GLAZER, T. JARROLD, J. ABRAHAM, C. THOMPSON AND V. R. HAWKINS 1953 The effect of vitamin B_6 deficiency induced by desoxypyridoxine in human beings. J. Lab. Clin. Med., *42:* 335–357.

WACHSTEIN, M., AND A. GUADAITIS 1953 Disturbance of vitamin B_6 metabolism in pregnancy. II. The influence of various amounts of pyridoxine hydrochloride upon the abnormal tryptophan load test in pregnant women. Ibid., *42:* 98–107.

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THIS NUMBER COMPLETES VOLUME 63

THE JOURNAL OF NUTRITION

VOL. 63 DECEMBER 10, 1957 No. 4

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PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America