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I hereby recommend that the thesis prepared under my supervision by Philip Delmar Adams

entitled THE OXYGEN UPTAKE AND THE COMPOSITION OF THE SKIN OF RATS IN VITAMIN G DEFICIENCY.

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by: Albert P. Mathews

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THE OXYGEN UPTAKE AND THE COMPOSITION OF THE SKIN OF RATS IN VITAMIN C DEFICIENCY.

A dissertation submitted to the

Graduate School

of the University of Cincinnati

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requirements for the degree of

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by

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The Oxygen Uptake and the Composition of the Skin of Rats in Vitamin G Deficiency.

I. Introduction.

During the past few years the physiology and the biochemistry of the skin have assumed greater importance as a subject of study, both from the point of view of medical applications and from the more practical point of view of its relations to environment. The study of the skin has been complicated, however, by the fact that little effort has been expended in arriving at some definite and reproducible measure by which the effects of any particular pathological state or of any particular external treatment could be ascertained.

The composition of the skin of both animals and humans has appeared from previous investigation to be widely variable from animal to animal as well as in different anatomical portions of the same animal, so it is difficult to assign any average values to any specific areas. Within the past ten years several investigators have begun an intensive study of the enzymes of the skin and their variation in some pathological states and the effects on the enzymes of various skin treatments. The difficulty of making complete extraction of enzymes from such a tough tissue as the skin makes the determination of their quantity

difficult. Different investigators have obtained very different results.

The respiration of the skin or of any tissue is probably a more accurate measure of its vitality and physiological activity than any other of its functions. If a measure of the respiration of the skin were made in different areas of the body, at different ages and in different diseases, then the physiological state of this important organ of the body could be determined. To measure the respiration it is only necessary to determine the amount of oxygen consumed and the amount of carbon dioxide produced by a known weight of tissue in a definite length of time. This determination can be made very accurately on very small amounts of tissue by the well known Warburg manometric method.

The present study is a comparison of the oxygen uptake of the skin of rats living on a complete diet with the oxygen uptake of the skin of rats of the same age when on a diet deficient in vitamin G (B_2). A study of the respiration of the skin in this vitamin deficiency was desirable since one of the prominent symptoms of this diseased state is loss of hair followed sometimes by dermatitis. This indicated a changed function in the skin. Moreover, it was believed that this avitaminosis might affect the skin respiration since various investigators are of the opinion

that all of the vitamins are involved in the oxidative processes of the body. While this has not been proved with respect to all the vitamins it is now definitely known that vitamins A, B_1, B_2, and C in some manner catalyse the oxidative processes of the body. It might be supposed therefore, that in a deficiency of vitamin B_2 there would be a decreased ability of the skin to consume oxygen.

The experiments to be described below show that in a deficiency of vitamin B_2 there is not only a decreased ability of the skin to utilize oxygen, but that there is also an increase in the water content and a decrease in the fat-free dry matter as compared to animals of the same age. It will also be shown that in normal rats there is a decreased ability of the skin to utilize oxygen in older as compared to younger normal animals. Determinations of phospholipid have shown that there is a quite rapid decline in the phospholipid content of the skin with age, but that there is no striking difference between normal and vitamin B_2 deficient rats in this respect.

II. Methods of Investigation.

1. Animals and diets:

The animals in Series I were secured locally. The animals used in Series II determinations were secured from the Sprague and Dawley Company. Those used in Series III were obtained from the Wistar Institute, Philadelphia, Pennsylvania. The animals were received at weaning time (28 to 30 days old) and then weighed from 40 to 60 grams. Only male animals were used throughout the study.

As soon as received the animals were divided at random into two groups and were placed in pairs in separate cages with open screen bottoms. The vitamin B_2 deficient diet of Bourquin and Sherman (6) was used for the production of vitamin B_2 deficiency. This diet consists of the following: extracted casein, 18% (freed of vitamins B_1 and B_2 by repeated extraction with 60% ethyl alcohol): F.R.L. salt mixture 4.0% (this mixture of Hawk and Bergeim as described in their "Practical Physiological Chemistry" page 693 (35) was substituted for the Osborne-Mendel salt mixture): cod liver oil 2%; butter fat 8%; potato starch on which had been dried the alcoholic extract of freshly ground whole wheat in such proportion as to supply the equivalent of 50 grams of whole wheat per 100 grams of air dry food mixture. The alcohol extract of the ground whole wheat was prepared as follows: 800 grams of freshly ground whole wheat were

shaken with 1,500 cc. of alcohol (80% by weight, allowing a water content of the wheat of 10%) for 1 1/2 hours. This was filtered through a Buchner funnel and the residue again treated with 1,000 cc. of alcohol. This was filtered again and the residue washed with about 300 cc. of alcohol. The filtrate and washings were evaporated under reduced pressure to a convenient consistency and finally dried upon starch, which was subsequently incorporated in the food mixture. It was found that the amount of supplement incorporated served to protect the animals from the polyneuritis of B_1 deficiency. The normal animals were fed a similar diet composed of non-extracted casein, powdered whole milk (to supply vitamin B_2), F.R.L. salt mixture, cod liver oil, and potato starch, together with the vitamin B_1 supplement from whole wheat.

2. Preparation of skin tissue and the determination of oxygen uptake:

Except with respect to diet all animals were treated alike. The determinations of the oxygen uptake were carried out in a room, the temperature of which could be held relatively constant at from 36° to 40° C.

The animals were stunned by a light blow on the head and immediately tied securely to an animal board. The hair of the back was clipped closely with scissors and then shaved after wetting with soft soap solution. Attempts

were made to dry shave the animals but this was found impossible without considerable injury to the skin. After shaving, the skin surface was washed thoroughly with warm tap water and dried by blotting with a paper towel. At this time the animals were killed by a sharp blow on the head. Sections of skin were then removed in a saddle shape from the back just below the scapula. Invariably, in order to avoid confusion and to facilitate the operation as much as possible, the section from the right side was used for the determination of the oxygen uptake, the section from the middle just above the spine was used for the determination of the water and dry matter content, and the section from the left side was used for the determination of total lipid and phospholipid.

The section from the right side of the animal was immediately washed with warm Ringer solution to remove any soap that might have escaped the preliminary washing. The skin was then stripped of any adhering fatty or connective tissue and placed flat between two pieces of paraffin-beeswax mixture (1/4 beeswax: 3/4 paraffin). In preliminary experiments numerous methods of slicing were tried, such as razor-sharp scissors, microtome, and free-hand with a razor, but all were found unsuccessful. After a little

practice it was found that by using the upper piece of paraffin-beeswax as a guide and slowly drawing it back, thin slices, sufficiently uniform, could be obtained very quickly with a razor. The purpose of the paraffin-beeswax mixture was to secure a composition hard enough to hold the skin firmly and of such texture that there was no chipping as with pure paraffin. The skin slices were immediately placed in warm Ringer-glucose solution in a covered Petri dish.

Before killing the animals the Warburg vessels were properly loaded with their solutions in order to reduce to a minimum the time elapsing from the stunning of the animal to the equilibration and closing of the manometers in the constant temperature water bath. The sections of skin as obtained above were removed from the Ringer solution in the Petri dish to the Warburg flasks by means of a small Ni-Cr hook, first touching the sections to a piece of clean filter paper in order to remove most of the Ringer solution.

Previous trials made it possible to estimate fairly closely the amount of tissue placed in each flask and subsequent weighings showed the amounts not to vary widely.

The flasks with the tissue were connected to their manometers and flushed several minutes with pure oxygen. The complete manometers were then placed in the constant temperature water bath with the manometers outside the bath

and the flasks completely immersed in the water. With the manometer arm stopcocks still open the flasks were shaken at 120 strokes of 5 cm. length per minute for 10 to 15 minutes for the volume of the gas contents to become constant. At the end of this time the level of the manometer fluid was brought to 150 mm. in each arm, the manometers closed, and the shaking continued for exactly 2 hours. At the end of 2 hours the level of the fluid in the right arm of the manometer was again brought to 150 mm. and the difference in the level of the right and left arm recorded as the difference in pressure caused by uptake of oxygen by the tissue. N/10 KoH in the central well removed the CO_2 as fast as formed.

The total time from the first stunning of the animals to the closing of the manometers was never more than 40 minutes nor less than 30 minutes. The temperature of the water bath was held at $37.5^{\circ} \pm 0.05^{\circ}$ C. This difference in temperature as well as any variations in the atmospheric pressure during the period of observation were allowed for by a blank determination which was invariably conducted with each determination in exactly the same manner as the experimental determinations, with the only difference that no tissue was placed in the flask. The reading of the blank determination was either added to or subtracted from the experimental determinations depending upon

whether the change of the blank was positive or negative.

After the reading of the manometers at the end of the 2 hour observation period the manometers were removed from the water bath and the tissue with the fluid poured into a weighed Gooch crucible containing a small piece of filter paper to serve as a pad. After sucking the tissue free of fluid it was washed quickly with 1 cc. of distilled water to remove the last of the Ringer solution. The tissue was dried at 95° to 100° C. for 2 hours and weighed again. On the average 30 to 50 mgm. of water free dry matter (equivalent to about 75 to 100 mgm. fresh tissue) was used in each flask. Because of the small amount of tissue and the thinness of the slices this drying period was found to give a constant weight, for drying 12 hours longer at 80° C did not change the weight.

The cubic millimeters of oxygen consumed divided by the weight in milligrams of water-free dry matter gave the cubic millimeters of oxygen consumed per milligram in 2 hours. The amount of fat-free dry matter in the manometer vessels was obtained from the value of total lipid determined separately, as was also the amount of phospholipid contained in the tissue of the vessel. The amount of oxygen per milligram of fat-free dry matter, and per milligram of phospholipid was also calculated. Because of the wide variation in the fat content of the skin it is believed that

the oxygen consumption per unit of fat free dry matter serves as a better basis of comparison, inasmuch as actual determination shows the fat-free dry matter to be more constant.

The Ringer solution which was used in the preparation of the tissue for the oxygen uptake and in the manometer vessels was prepared as follows:

NaCl, recrystallized,	9 grams per liter	1,000 cc.
HCl, recrystallized,	11.5 grams per liter	20 cc.
$CaCl_2$	12.2 grams per liter	20 cc.

To each 9 cc. of this Ringer solution was added 1 cc. of phosphate buffer mixture, pH 7.4 . The phosphate buffer mixture consisted of 2 cc. KH_2PO_4 (9.078 grams per liter) plus 8 cc. of $Na_2HPO_4.2H_2O$ (11.876 grams per liter). To the completed solution thus prepared was added pure glucose to give a concentration of 0.2%. The Ringer solution before the addition of the buffer mixture was found to have a pH of 6.9 measured by the quinhydrone electrode. The standard buffer mixture, which according to Clark's "The Determination of Hydrogen Ions" should have a pH of 7.4 was found in successive separate mixtures to have a pH varying from 7.3 to 7.4 . The completed Ringer-phosphate-glucose solution was found to have the same pH as the buffer. After several of the preliminary trials with this method of determining the oxygen uptake of tissues, the Ringer fluid remaining after the oxygen uptake was determined was collected from

several manometer vessels and its pH measured. It was found not to have changed appreciably from the initial value. In order to test the effect of slight variations in the pH, determinations were made at pH 7.6, 7.3 and 7.1. No appreciable variation in the oxygen uptake was noted. This is in agreement with the findings of Warburg that variations in the pH of the fluid bathing carcinoma tissue Ringer solution in vitro) did not appreciably alter the oxygen uptake.

The manometer fluid used in these experiments was the Brodie Fluid recommended by Warburg (73). This solution consists of the following: water 500 cc., NaCl 23 grams, bile salts 5 grams. Bacterial and mold growth is prevented in this solution by the addition of just sufficient alcoholic solution of thymol to produce a faint smell. Addition of a small amount of Bismark Brown facilitates reading of the fluid level in the manometers. After determination of the specific gravity of this fluid with a 25 cc. pycnometer bottle it was found necessary to add a little water in order to bring the specific gravity to 1.034. A fluid of this specific gravity is used to increase the sensitivity of the manometers, 10,000 mm. of Brodie Fluid being equal to 760 mm. of mercury. The sensitivity of the manometers is thus slightly more than 13 times that of manometers in which mercury is used.

The manometers and flasks were standardized by the use of mercury as follows: the flask was filled with clean mercury and placed on the manometer, just enough mercury being used to bring the mercury column into the manometer at some arbitrary point, which is marked with a pencil. The flask with the mercury in the manometer arm is removed, and mercury is then placed in the manometer to extend from this point to the 150 mm. level in the right manometer arm. This mercury is then added to the mercury in the flask and from the weight of the mercury the volume of the flask and the manometer arm down to the level of the manometer fluid is obtained. This volume must be known so that the relation between the change in pressure and the volume of oxygen can be determined, inasmuch as this apparatus gives the change in pressure at constant volume as oxygen is consumed by the tissue. The change in pressure must then be changed to cubic millimeters. This is accomplished as follows, using the calculations as given in the methods of Warburg (73):

	Cu. Mm.
Total volume of vessel and capillary to the 150 mm. mark as determined by mercury	16,975
Volume of KOH in central well	400
Volume of Ringer solution	3,000
Total volume of fluid	3,400
Volume of free gas space (total volume minus fluid)	13,575

$$\frac{Vg \times \frac{273}{T} + Vf.a}{10,000} = KO_2$$

K = manometer constant.
Vg = volume of gas to 150 mm.
T = absolute temperature of bath.
Vf = total volume of fluid.
a = Bunsen coefficient of solubility for oxygen in water at 37.5° C.= 0.024.

$$\text{Therefore } KO_2 = \frac{13,575 \times \frac{273}{310.5} + 3,400 \times 0.024}{10,000} = 1.201.$$

Thus a change of 1 mm. pressure is equivalent to 1.201 cu. mm. of oxygen at 0° C. and 760 mm. pressure.

3. The determination of water and dry matter.

The water content was determined on a portion of skin taken from the region overlaying the spine. From 0.4 to 1.5 grams of skin were dried at 95° to 100° C. for 2 hours. The loss in weight was taken as water loss. The dry residue served as the determination of water-free dry matter. Drying over night at 80° C. did not alter the value.

4. The determination of total lipid and phospholipid.

The total lipid was determined by a modified method of W. R. Bloor (8) which was as follows: An amount of skin not exceeding 2.0 grams and sometimes as little as 0.5 grams, previously exactly weighed in a glass stoppered weighing bottle, was snipped with scissors into as small

fragments as possible, being held with forceps during the cutting. The pieces were dropped directly into a 125 cc. Erlenmeyer flask and were covered with about 30 cc. of ethyl ether. The tissue with the ether was let stand at room temperature till the following morning, when the ether was pipetted off with a rubber bulb pipette and transferred to a 100 cc. beaker. To the tissue remaining was then added 30 cc. of ether-alcohol mixture (2 parts of ether to 1 part of 95% alcohol) and the whole warmed to the boiling point on the steam bath for about 30 minutes. This was repeated with 2 additional portions of 30 cc. of ether-alcohol mixture, each time after heating the extract being pipetted into the same beaker as previously. Following the ether-alcohol extraction 3 or 4 similar extractions were made with boiling 95% alcohol. All the extractions were combined in the same beaker as before and the solvent evaporated on the steam bath by the aid of a gentle current of air. After all the solvent had been evaporated the fatty residue was dried in a desiccator under a high vacuum for 4 to 6 hours over concentrated sulphuric acid or anhydrone. The dry residue was then extracted with 5 or 6 portions of petroleum ether. The petroleum ether used in this extraction had been shaken with concentrated sulphuric acid and let stand at least 24 hours with occasional shaking, after which the ether was decanted into a distilling flask

with several grams of solid NaOH and redistilled. The petroleum ether extract of the fatty residue was filtered into a 50 cc. volumetric flask, made up to volume and two aliquots taken as follows:

(1) Twenty-five cc. measured into a 50 cc. centrifuge tube for the determination of phospholipid which will be described below.

(2) Twenty cc. measured into a weighed 25 cc. weighing bottle for the determination of total lipid. This aliquots was evaporated to dryness on the radiator, and finally in a vacuum desiccator for several hours. The weight of the residue times 2.5 was taken as the total lipid in the section of skin weighed from the left side of the animal.

Aliquot (1) was evaporated to dryness and the phospholipids determined as follows: To the dry fatty residue (after washing down the sides of the tube with not more than 1 cc. of ethyl ether) was added 10 cc. of acetone and 3 drops of saturated magnesium chloride in 95% ethyl alcohol. The mixture was stirred gently by rotation and cooled under the tap after which it was immediately centrifuged. The acetone, which was invariably water-clear after centrifuging, was decanted and discarded. The phospholipid with the small amount of lipids which remained was redissolved in ethyl ether with washing of the sides to prevent as much

as possible any of the lipid material remaining on the sides of the centrifuge tube. The ether was evaporated on the sand bath. From this point the determination was completed by the method of Fiske and Subbarow (24) which is as follows: To the phospholipid residue in the centrifuge tube was added 2.5 cc. of 10% sulphuric acid. The tubes were then gently heated either on a sand bath or in an oven until most of the water had evaporated and charring had begun. They were then gently heated over a microburner until no further charring was evident and then somewhat more strongly until the appearance of dense white fumes. After permitting to cool slightly 3 or 4 drops of concentrated nitric acid were added and the heating continued until the contents of the tube were colorless. The contents of the tubes were then transferred to 25 cc. volumetric flasks with about 15 cc. of distilled water. To the flasks was then added 2.5 cc. of 2.5% ammonium molybdate, followed by 1 cc. of aminonaphthol sulphonic acid reagent which reduces the phosphomolybdate to a blue color. This was then compared in a colorimeter with a standard solution prepared from potassium acid phosphate. From the colorimeter reading the amount of phosphorus in the skin sample was calculated. Taking the molecular weight of lecithin as 777.53 and the atomic weight of phosphorus as 31.027 then it is evident that 1 milligram of phosphorus is

equivalent to 25.06 milligrams of lecithin. The phosphorus of the total phospholipids has been calculated as lecithin although it is realized that the substance isolated by this method is the whole group of phospholipids, chief of which, however, are lecithin and cephalin.

Chart I shows a representative complete analysis of all the skin sections indicating the manner in which all the values reported in this paper were derived.

(Insert Chart I.)

III. Review of Literature.

Probably the earliest American work suggesting a growth-promoting substance in natural foodstuffs is the work of T. B. Osborne and L. B. Mendel. In 1913 (56) they developed more fully a previous suggestion of the presence in protein-free milk of a substance important for growth differing from any of known constituents of the diet. In the few years following the term vitamins had come into general use and the antineuritic vitamin B had been ascertained to exist. Up to 1919, however, it was generally accepted (cf. the review of H. H. Mitchell (54), that the antineuritic and growth-promoting vitamins were identical. Until 1926 the evidence for the non-identity of the two vitamins grew slowly when greater impetus was given the

Chart I.

Calculations involved in the determination of O_2 uptake and analysis of rat skin.

Oxygen Uptake

Time	5 Blank	1	2	
1:53	149.2	150.2	149.0	
3:53	162.0	135.7	119.8	
	+12.8	-14.5	-29.2	Manometer change in mm.
	1.19	1.20	1.18	Manometer constant.
	+15.23	-17.40	-34.45	Observed volume change cu. mm.
		+15.23	+15.23	
		-32.63	-49.68	Observed vol. + Blank vol.= Total vol.
Note: Mm. pressure change x manometer constant = cu. mm. O_2 at 0° C., 760 mm.		1.11	1.13	Cu. mm. O_2/mg. H_2O-free dry matter.
		2.49	2.54	Cu. mm. O_2/mg. fat-free dry matter.
		111.0	113.4	Cu. mm. O_2/mg. phospholipid.
		7.4726	7.6304	
		7.4432	7.5866	
		29.4	43.8	H_2O-free dry tissue in mgm.
		16.3	24.3	Fat of H_2O-free tissue in mgm.
		13.1	19.5	Fat-free dry matter in mgm.
		.294	.438	Mg. phospholipid.

Water and dry matter content

24.8164
23.8227
993.7 Mg. fresh tissue.

24.2200
23.8227
397.3 Mg. dry tissue.

$\frac{397.3}{993.7} \times 100 =$ 39.98% Water-free dry matter.
60.02% Water of fresh weight.

Chart I.

Calculations involved in the determination of O_2 uptake and analysis of rat skin. (continued)

Total Lipid

1769.3 Mg. fresh tissue.
.3998 % dry matter.
707.4 Mg. H_2O-free dry matter.

392.5 Mg. total lipid.
314.9 Mg. fat-free dry matter.

157.0 Mg. total lipid in 20 cc.
2.5
392.5 Mg. total lipid in tissue.

$\frac{392.5}{1769.3} \times 100 = 22.18\%$ fat of fresh weight.

$\frac{392.5}{707.4} \times 100 = 55.48\%$ fat of H_2O-free dry matter.

$\frac{707.4 - 392.5}{1769.3} \times 100 = 17.80\%$ fat-free dry matter of fresh weight.

Phospholipid

.142 Mg. P in 25 cc. aliquot.

.142 x 2 = .284 mg. P in tissue sample.

.284 x 25.06 = 7.12 mg. phospholipid.

$\frac{7.12}{1769.3} \times 100 = .402\%$ P-lipid of fresh weight.

$\frac{7.12}{707.4} \times 100 = 1.00\%$ P-lipid of H_2O-free dry matter.

$\frac{7.12}{707.4 - 392.5} \times 100 = 2.25\%$ P-lipid of fat-free dry matter.

$\frac{7.12}{392.5} \times 100 = 1.81\%$ P-lipid of total lipid.

subject by the work of J. Goldberger et al (26) in which they suggested the non-identity of the two factors and used the term P-P (pellagra-preventive) to designate the factor other than the antineuritic. From this time on evidence grew more rapidly that the antineuritic and growth-promoting factors were separate entities, although there is still some doubt as to the identity of human pellagra and vitamin G deficiency in animals.

At about the same time that the work of Goldberger and his associates appeared M. I. Smith and E. G. Hendrick (67) reported what is probably the first clear-cut evidence that vitamin B was really a complex rather than a single substance. They showed that a diet adequate in all other respects excepting the B vitamins was not made complete with Seidell's vitamin B picrate, nor with autoclaved yeast, thus indicating at least two components of the complex.

Since the work of these investigators numerous studies have confirmed the complex nature of vitamin B, until at the present time there is good evidence for B_1, B_2 (or G), B_4, and B_6.

However, in spite of numerous attempts to refine the feeding technique the development of vitamin G deficiency has been attended with wide variability and great uncertainty. Certain investigators (27, 6, 64) have on their diets observed loss of weight, development of

dermatitis with loss of hair and desquamation, soreness of feet and stomatitis. Other investigators have been unable to secure uniform results (2, 62), only 30 to 50 per cent of the animals developing dermatitis. More recently various devices have been introduced to secure more uniform development of dermatitis, such as irradiation of yeast and of rice polishings to destroy the anti-dermatitis factor, and the use of sources of anti-neuritic vitamin, free from all but rraces of G, such as white corn, rice polishings, various adsorbates from yeast (Fuller's earth, Norit, etc.).

In an excellent review of the vitamins by the Medical Research Council of London (49) the following conclusion is drawn after examination of the literature on vitamin G: "it may be remarked that dermatitis associated with vitamin G deficiency appears to be produced in the rat with some uncertainty. In none of the experiments just recorded did more than a proportion of animals show skin lesions, and several investigators have failed to observe any characteristic symptomatology beyond, perhaps, a condition of general wretchedness. Those who have done much work on vitamin G are puzzled by the changeable nature of the lesions occurring in the vitamin G deficient animal and by the frequent failure to produce any characteristic skin symptoms. Complete cessation of growth

without any accompanying decline in weight is, however, a constant feature of young rats provided with vitamin B_1, but deprived of G". In a still later review of the literature L. J. Harris (33) arrives at about the same conclusion: that the symptoms of vitamin G deficiency are not well understood and that dermatitis is quite variable even on the same diets by various investigators.

To account for the variable symptoms on vitamin G deficient diets various investigators have suggested that factors in the environment or in the diet (other than vitamin G) may be responsible. W. D. Salmon, I. M. Hayes and N. B. Guerrant (63) found a large coccus constantly associated with the lesions of vitamin G deficiency and postulated that rat pellagra is the result of microbic infection plus dietary deficiency. Violet Reader (59) devised a diet containing sucrose and found that when the cane sugar was omitted from the diet pellagra did not develop. She also observed a seasonal variation in the development of pellagra, but this has never been confirmed. More recently, N. B. Guerrant and R. A. Dutcher (28) have attained greater success in the production of dermatitis by the feeding of diets high in sucrose (60 to 70%), believing the development of the symptoms to be dependent on high caloric intake. H. C. Sherman and I. A. Derbigny (65) found a definite relationship between protein supply and vitamin G deficiency, although

other investigators have obtained opposite results with respect to the protein content of the diet (21, 58, 38). L. N. Ellis (18) found inferior growth to be associated with higher concentrations of vitamin G, while Minerva Kellogg and W. H. Eddy (41) found that dermatitis developed more uniformly when vitamin G was low and B_1 was high, the ratio of the two being more important than the absolute amount of G. Remp and Bing (61) present evidence that inanition is a significant feature of the syndrome of vitamin G deficiency, although assuming inanition to be a factor still does not explain the discordant results obtained by investigators generally.

The wide variability in the symptoms of vitamin G deficiency can be most easily explained on the supposition that vitamin or vitamin-like factors other than vitamin B and G are concerned. Such factors have been suggested by various investigators. Chick and Copping (9) suggested the existence of a third factor necessary for growth which they called factor Y. Hunt (39) and Hunt and Wilder (40) presented evidence for a third factor different from that of Chick and Copping in that it was alkali-thermolabile. Violet Reader (60) and others (44, 11, 32) had also postulated a third factor found in whole wheat; since rats on diets adequate in vitamins B and G declined in weight, but made striking gains when only a very small quantity

of whole wheat was added to the diet. This vitamin was designated as B_4. R. Kuhn, Paul Gyorgi and Th. Wagner-Jauregg (46) stated that vitamin G could be separated into two factors, one of which they called the skin factor (vitamin H, or Haut-faktor). Hogan and Richardson (38) stated that corn starch or its alcoholic extract would cure dermatitis but would not permit resumption of growth.

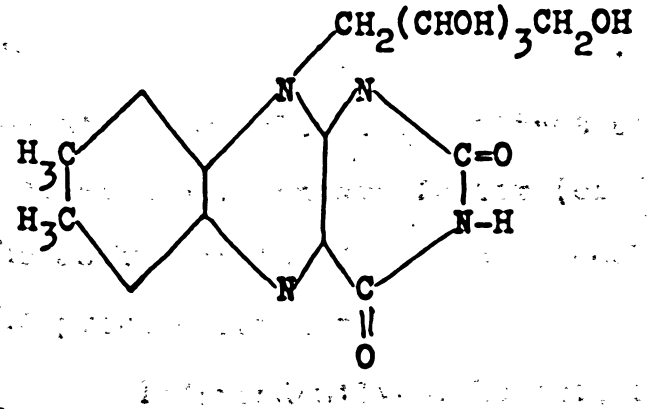
Recently Paul Gyorgi (29) found that symptoms of pellagra could be produced in rats by a diet adequate in vitamins B and G. The animals could be cured by alkaline autoclaved marmite (in which vitamin B_4 is destroyed). He concluded that vitamin G had been shown to consist of two components: the real vitamin G (flavin) and vitamin B_6, as he tentatively designates the new fraction.

The greatest clarification of the nature of vitamin G and G deficiency has come since 1933 consequent upon the isolation and purification of the flavines. Warburg, O. and W. Christian (74) succeeded in obtaining in pure crystalline form from yeast a yellow pigment which acted as an oxidation catalyst. They called this the oxidation enzyme, but did not identify it as a vitamin, although later work by others (30) have shown it to act as vitamin G.

In 1933, P. Ellinger and W. Koschara (17) announced the isolation of a new group of animal pigments for which they proposed the name lyochromes. At about the same

time R. Kuhn and his co-workers announced the isolation from such vitamin G containing sources as milk whey, egg white, and animal tissues, green fluorescent pigments for which the name flavines was suggested. In a later extended series of papers Kuhn and others (48) showed that the flavines could act as biological oxygen carriers, and that when supplemented with small amounts of boiled yeast extracts from which the flavines had been removed, could act as vitamin G for rats in amounts as small as 5 gamma per day. The flavines alone, however, did not so act.

Continuing his work on the constitution of lactoflavine (the flavin from whey) and attempts to synthesize it, Kuhn has suggested the probable structure and by synthetic methods has obtained flavines identical in every respect to the naturally occurring flavines. The structure, according to R. Kuhn and Weygand (47) is as follows:



or $C_{17}H_{20}N_4O_6$

The only portion of the molecule the authors believe to be in doubt is the pentose side chain, although it is possible that this chain can be varied in its configuration without

affecting the action as vitamin G. Tests with this synthetic flavine on vitamin G deficient animals have been uncertain, several investigators (5, 20) having reported it unable to act as vitamin G. It has also been found unable to act as a biological oxidation catalyst in vitro without the addition of liver pulp (72).

Up to the time of Kuhn's preparation of the pure flavines the only factor of the vitamin B complex obtainable in pure state was vitamin B_1, the anti-neuritic vitamin, although its cost and the difficulty of its preparation made its use in many laboratories almost prohibitive. Nevertheless, the greatest single advance in the clarification of the problem has come recently with the use of highly purified materials, such as crystalline vitamin B_1 and the flavines. Lela Booher and co-workers (5), cited above, have shown that crystalline vitamin B_1 and flavines could not prevent vitamin G deficiency (i.e. permit continuous growth in rats) without the addition of a very small amount of 80% alcoholic extract of whole wheat. Evidently another supplementary factor (or factors) is required for the action of the flavines. Booher has not yet developed the problem further.

Independently of Booher, and at about the same time, several workers in England have made the same observation that Booher has made but they have developed it in greater

detail. Chick, Copping and Edgar (10) point out that earlier investigations with highly concentrated materials were somewhat in error, since with still greater purification it has become evident that crystalline vitamin B_1 plus flavine do not supply the full function of the B complex, but that some additional factor is required. It was at first thought by Kuhn and others that the missing factor was vitamin B_4, but it has since been shown by Paul Gyorgi (29) that the missing factor was not Reader's vitamin B_4. Chick and her co-workers draw the following conclusions from their data: (1) vitamin G is composed of two components: flavin and a supplementary substance which is apparently identical with the factor Y of Chick and Copping (9) and with the vitamin B_6 of Gyorgi (29). They therefore suggest the term vitamin B_2 to designate the two factors together (flavin plus vitamin B_6) and that one constituent be called simply flavin and the other vitamin B_6 as already suggested by Gyorgi. Chick and co-workers also believe two types of dermatitis to exist: a florid type, or true rat pellagra and a generalized type. Their conclusions regarding this and the effects of the two constituents of the vitamin complex are irregular and not very conclusive.

Paul Gyorgi (31) in a further analysis of the problem confirms the work of Chick et al. He believes the pellagra preventing substance to be the new fraction,

vitamin B_6, which is also needed to demonstrate the growth-promoting action of the flavin. Neither the flavin nor the supplementary substance alone possess any growth-promoting action. Furthermore, egg-white, although it is rich in flavin, has no anti-pellagra activity.

Further studies using the purest obtainable materials promise to completely clarify the problem and to lead to further advances in the field of vitamins generally. Assuming the work of these latter investigators to be correct it is possible to explain entirely the wide variations observed by various investigators in the past. It is also possible on these assumptions to explain the egg-white dermatitis which has been extensively investigated by M. A. Boas (3), Parsons and co-workers (57), and by Fixsen (25).

The persistently wide variations in the results of various investigators of vitamin G deficiency leads to the conclusions that they have all produced by their individual technique different degrees of purity in the food materials. Under ordinary conditions animals are able to withstand wide variations in the protein, fat, carbohydrate and mineral content of foods. Hence, it seems improbable that these factors can be fundamental to the production of vitamin G deficiency. The assumption has been held that vitamin G was a simple substance and like the other vitamins required no additional substances for its activity which were not already

present in the animal body. Thus its complex, or manifold, nature has escaped detection until the highly purified vitamin B, and the pure flavines were available. For the most part, vitamin research in the future will proceed in proportion to the adoption and use of pure isolated vitamins instead of natural sources in foods which thus may introduce unknown substances.

The mode of action of the vitamins and their diverse physiological effects is not completely clear, but, with the exception of vitamin D, the well defined vitamins (A, B_1, B_2 or G, and C) have been shown by abundant evidence to function in some manner in cell oxidation.

The first of the vitamins shown to be involved in the oxidative processes of the cell was the anti-neuritic vitamin B_1. In 1918 Dutcher (15), and again in 1921 Findlay (23), reported a fall in catalase and glyoxalase of B_1 deficient pigeon tissues. Abderhalden and co-workers (1) and W. R. Hess and co-workers (37) showed a reduced intensity of tissue oxidation in B_1 deficiency. Terroine and Roche (70) and Drummond and Marrian (16) failed to find any decrease in tissue oxidation. Kinnersley and Peters (42) have found a lowered oxygen uptake and increased lactic acid content of the brain of B_1 deficient pigeons. They have more recently shown (43) that the lowered oxygen uptake can be restored by the addition of vitamin B

concentrates in vitro, and that the abnormality is specific for the glucose-lactate systems. Stare and Elvehjem (68) have confirmed the lowered oxygen uptake of the cerebellum in chicks and rats in avitaminosis B_1.

Since the isolation of vitamin C and its identification as hexuronic (ascorbic) acid numerous papers have shown it to be strongly reducing and reversible in its action. Harrison (34) and H. von Euler and E. Klussmann (19) have shown that scorbutic animal tissues have a lowered oxygen uptake which is restored in vitro by the addition of ascorbic acid, while the addition to normal tissue does not increase the oxygen uptake. Szent-Gyorgyi and K. Vietorisz (69) have shown that hexuronic acid increases the action of potato oxidase about fifteen times.

Even before the identification of beta-carotene as the precursor of vitamin A it was known that vitamin A was very readily oxidized. Carotene itself is a highly unsaturated compound which has been shown to act as a transporter of oxygen. W. Franke (22) has shown that carotinoids, including carotene, are able to catalyse the oxidation of unsaturated fatty acids. The effect is enhanced by hemin.

Investigations on flavines have shown them capable of reversible oxidation-reduction. Upon removal of oxygen the yellow color disappears, but appears again on shaking

with molecular oxygen. They are decolorized by yeast, muscle or other tissue hash and the decolorization is accelerated by the addition of lactates, pyruvate, succinate, citrate and aldehyde (71). In a later investigation, however, it is stated (72) that the flavines are natural hydrogen acceptors in the dehydrogenation process in muscle, but require complementary substances in the nature of activators.

Investigations of tissue metabolism have revealed a number of oxidation-reduction systems which may be mutually interdependent. It is improbable that only one system of oxidation-reduction exists, and therefore, the measured oxygen uptake or carbon dioxide production of a cell is the sum total of the changes brought about by all the systems. It is thus impossible at the present time to give the vitamins a definite place in the metabolic systems of the cell. It is significant, however, that vitamins A, B_1, B_2 (or G) and C have been shown to function in some manner in cell oxidations and that a deficiency of these vitamins results in retarded growth, loss of appetite and decreased metabolism. Although it has not been shown to do so, it is probable that vitamin D is involved in a similar function in the growth of bone tissue. Vitamin E, the anti-sterility vitamin, is known to be involved in fetal development and in the normal functioning of the

testes, in both of which metabolism is known to proceed at a high level.

IV. Experimental Observations.

1. The effect of age on the oxygen uptake and composition of the skin.

In preliminary experiments on the oxygen uptake of the skin it was found that there was a marked difference in the amount of oxygen absorbed by the skin of very young as compared to very old animals. A separate extended series, varying only the age, has not been determined (other than the animals of Series III) but in preliminary observations it was found that very young rats 1 to 5 weeks of age varied in oxygen uptake from about 6 to 10 cu. mm. per mg. of water-free dry matter. Table I presents several animals from the control group of Series III, together with several other animals on which analyses were made in the process of testing the methods and technique. These animals varied in age from a suckling of 10 days to a mature animal of 7 months.

(Insert Table I.)

The oxygen uptake falls regularly from 9.5 cu. mm. per mg. water-free dry matter at 10 days to 1.00 at 210 days. The preliminary series, in which no analyses were made, however, indicate that the skin of mature animals

Table I.

Animal Number	Age in days	Oxygen Uptake in cu. mm. per mgm. water-free dry matter/2hours.	Water content of fresh weight, %	Water-free dry matter of fresh weight, %	Fat-free dry matter of fresh weight, %	Total lipid of fresh weight, %	Phospholipid of fresh weight, %	Phospholipid of total lipid, %	Phospholipid of water-free dry matter, %
Suckling	10	9.80	67.80	32.20	11.50	20.66	1.12	5.41	3.47
24 C	36	7.20	68.53	31.47	20.86	10.60	0.807	7.61	2.56
5 R	79	3.50	59.10	40.90	25.60	15.30	0.475	1.85	1.16
6 A	105	2.98	59.73	40.27	27.84	12.43	0.546	4.39	1.35
A	180	1.45	62.62	37.38	29.50	7.85	0.329	4.20	0.89
O	210	1.00	63.47	36.53	32.30	11.17	0.494	11.90	1.35

(not carefully selected for age, parentage, kind of food upon which they were raised, etc.) may vary in their oxygen uptake from as low as 0.5 to as much as 2.00 cu. mm. per mg. water-free dry matter. In spite of this variation in mature animals, it will be seen that the degree of oxygen uptake falls quite rapidly to about the age of 50 days, after which it may vary within the limits stated, which is evidently far lower than young, rapidly growing animals. No determinations have been made on animals in the last stages of senility.

The water-free dry matter, which includes lipids, does not show quite as regular change with age as does the fat-free dry matter. The water-free dry matter will vary with the amount and type of feeding. The water content of the skin shows an appreciable fall with age from 67.80% of the fresh skin to an average value of about 60% for those animals from 79 to 210 days old. At the same time the fat-free dry matter (as % of the fresh skin) increases regularly from 11.50% at 10 days to 32.30% at 210 days.

Apparently the dry matter of the skin increases to a greater extent than the dry matter of other organs. Lowrey (51) determined the dry matter (including hair, ears and nails, and lipids) of the whole skin and of some of the internal organs of rats of varying ages. He has found rat skin at birth to contain 12.3% dry matter which rapidly

increases to 41.1% at 20 days and then more slowly to 45.5% at 12 months. Although the analyses in the present thesis do not show such a high content of dry matter (having been made on the shaved skin and do not include hair and nails) it is evident that the figures are parallel, and indicate a much higher dry matter content than the internal organs. Taking Lowrey's figures for comparison: for the liver at birth he found 19.4% dry matter which rose gradually to 26.0% at 12 months; for the musculature he found 10.7 at birth which rose gradually to 23.8% at 12 months. Lowrey's striking increase in dry matter of the skin from birth to 20 days is probably due in part to the increase in hair since rats are born hairless and show appreciable hair growth within 2 weeks after birth. Regardless of the absolute amounts it is shown by my own analyses that the dry matter of the skin almost doubles between 10 to 36 days, whereas by the data of Lowrey the muscle dry matter just little more than doubles in 12 months, while the liver dry matter increases in 12 months only about one-third more than that at birth.

It has long been known that the water content of tissues decreases with age. Donaldson (14) showed that the water content of the brain and spinal cord of rats is linked with age and is not readily modified by nutritive conditions. Burger and Schlomka (Z. ges. Exptl. Med. 58:

710-24, 1928) analysed the skin of fresh cadavers and found the water content to decrease up to 70 years of age. They believe the loss of water to be a very basic phenomenon of the ageing of tissues. On the basis of water content, then, one might say that the skin ages faster than other organs, since the dry matter increases to a greater extent than in other organs with simultaneous and proportional loss of water.

On the other hand, the phospholipid content of the skin declines with age. In very young animals it is 1.12 per cent of the fresh weight, or 3.47 per cent of the water-free dry matter. At 79 days it falls to an average level of about 0.5 per cent of the fresh weight and about 1.00 per cent of the water-free dry matter. Inasmuch as the water-free and fat-free dry matter increase markedly with age, then this decline of phospholipid to a relatively constant level may be interpreted in several ways, depending upon which theory of the function of the phospholipids is accepted. Whether the theory that they are intermediate stages in fat metabolism, or whether they are oxygen carriers is selected, it is still remarkable that there appears to be a conservation of the phospholipid, which seems likely in view of the decline in phospholipid with the increase of dry matter.

Since the work of Mayer and Schaeffer (52) the

weight of evidence seems to favor their view-point. They detected no significant effect of fasting, overfeeding or type of diet on the amount of phospholipid in the various tissues. The remarkable constancy and the universal distribution of the phospholipids led them to conclude that the phospholipids have some more general and more fundamental purpose than as a fuel. They suggested that they function as oxygen carriers.

The data presented for the oxygen uptake compare also favorably with the observations of Loebel (50) who found for the skin of newborn white mice a value as high as 11.6 cu. mm. per mg. of water-free dry matter per 2 hours, and for the skin of one newborn white rat 7.0 cu. mm. per 2 hours. The value found for mature rat skin is much lower than that found by Klopstock (45) for fresh human skin (adult). He found an average value of 4.2 cu. mm. per mg. of water-free dry matter per 2 hours, with values ranging from 2.4 to 10.2 cu. mm.

Hawkins (36) states that the metabolism of isolated tissue (liver) of rats is similar at different ages except in the very young. Meyer, McTiernan and Aub (53) find that the age of mice within wide limits has little effect on the metabolism of isolated tissue. The present investigation confirms their general conclusion: that in very young rats the oxygen uptake may be as high as about

10 cu. mm. per mg. of water-free dry matter per 2 hours, the value dropping at 4 to 6 weeks to a relatively constant value of about 1 cu. mm. From this age on, however, the value may vary in non-uniform animals as previously stated, from 0.5 to as much as 2.0 .

While the preliminary series of animals were taken from several different sources and in many cases the ages were not accurately known, the observations indicated that a decreased oxygen uptake was to be expected with age. This observation is not surprising since it is well known that the basal metabolism of the human body as a whole decreases after about 20 years of age.

2. Preliminary experiments with vitamin G deficient animals.

Series I.

In this series the animals were received several months before being placed on the diets. At the time of placing on the vitamin G deficient diets the animals were three months old. Growth of these animals was definitely sub-normal, although no other symptoms were evident even after continuing on the deficient diets for three months. None of 30 animals showed appreciable loss of hair, except for a slight thinning on the fore-paws and about the ears.

In a study of the effect of vitamin G deficiency on rats of different ages, Daniel and Munsell (12),

observed young animals to gain weight for a time, while older animals either remained at a constant level or declined in weight slowly to a constant level which was maintained over periods as long as a year. Very young animals, however, were more subject to development of alopecia than older animals.

The oxygen uptakes determined on these animals led to no conclusion. No difference between these and normal animals was apparent, the variations in the animals on the deficient diet being wide and there being no regular variation with length of time on the diets.

These preliminary trials led to the conclusion that one,or more,of several factors was involved in the wide variability and inconclusiveness of the results: (1) that the animals were too old when placed on the diet, and hence (2) were able to resist the deficiency by their greater bodily stores of vitamin C, or that (3) some factor in the technique was at fault, or (4) that the animals were not uniform in respect of percentage, etc.

In order to test the third factor several tests were made on the effect of variations in the pH of the medium in which the tissues were bathed during the determination. The results of this test follow below.

3. The effect of pH on the oxygen uptake of the skin in vitro.

Three media were prepared for the determination of O_2 uptake differing only in the pH, by adding buffer mixture to portions of a single sample of Ringer-glucose solution. The pH of each portion was then determined by the quinhydrone electrode. The following results were obtained:

				Deviations as % of the value at pH 7.3	
pH	7.1	7.3	7.6	7.1	7.6
Rat 1	0.511	0.517	0.569	-1.1	+10.0
Rat 2	0.427	0.430	0.493	-0.7	+14.0

These values are the oxygen uptake in cu. mm. per mg. of water-free dry matter per 2 hours. Normal animals 2 years old and weighing approximately 350 grams were used. Since duplicate samples in this determination may show as great a variation as 10 per cent it is evident that the variation attributable to possible variations in the pH is negligible. Subsequent determinations have shown the day to day variation in the pH of freshly buffered Ringer-glucose solution to be less than 0.1 pH, and that a 2-hour period of respiration of the tissue in the buffered Ringer-glucose solution does not appreciably affect the pH.

O. Warburg (73) tested the effect of pH on liver

and carcinoma tissue in vitro and found no appreciable effect of variations from pH 6.5 to 8.5. This is not true, however, of sea urchin eggs.

4. Series II. Young animals on vitamin deficient diet.

Table I presents data on a new group of young animals. These animals, all the same age (46 days), were purchased from Sprague and Dawley Company. They were placed on the deficient diet as soon as received.

Of 14 animals on the deficient diet, 5 developed more or less symmetrical alopecia, the areas being completely denuded. No other symptoms appeared other than sub-normal growth, 9 of the 14 showing a slight gain in weight and 5 a slight loss in weight. Although, as recorded in the Table, none of the animals were found to have appreciable deposits of intravisceral fat, they were still not emaciated.

When this series was begun the question arose as to whether stomach ulcers might be present and thus account for some of the symptoms. Of 14 animals only two were found to have stomach ulcers. It is concluded that this is not a factor in the development of symptoms.

According to P. L. Day and co-workers (13), and O'Brien (55), the development of bilateral cataract is a more dependable symptom of vitamin G deficiency than any other. According to them cataract appears in 96% of

animals in vitamin G deficiency. The cataract is visible to the unaided eye, but may be detected at an earlier age by the use of an ophthalmoscope. In the animals of the present Series II only 2 of 14 developed cataract. In Series III, to be described below, none developed cataract.

The oxygen uptake of 14 deficient and 6 normal rats is reported in Table II. It will be seen that the oxygen uptake of the deficient animals does not fall as low at any time as that of the normal animals. This was just the contrary of the expected result.

In the course of this series (II) and in the preliminary experiments of Series I, it was noted that the skin of deficient rats was always much thinner (about one-third as thick) than that of normal animals of the same age but of greater weight or of the same weight. Examination of microtome sections of deficient and normal skins showed the germinal layer, which is presumably the most active, to be of the same thickness in the two. It was thought, therefore, that in normal animals the physiologically most active cell layers might be "diluted" with such relatively low respiring constituents of the skin as connective tissue, lipids, etc. as to make a true comparison of their relative activities difficult, since the oxygen uptake was calculated on the water-free dry matter of the tissue. If the non-respiring dry matter were

present in larger amounts in the one case than in the other, the respiration would appear to be correspondingly lower. The skin of the normal animals was observed to be very fat, in some cases causing the appearance of fat globules on the surface of the Ringer solution when the skin was cut into thin sections.

It was decided, therefore, to undertake a new and somewhat larger series of animals, which is presented below as Series III.

(Insert Table II.)

5. Series III. The oxygen uptake and composition of vitamin C deficient and normal rat skin.

In Table III and Figures I and II is presented the oxygen uptake, the dry matter content, the water content, the total lipid content, and the phospholipid content of 28 deficient rats and 14 normal rats.

The rats used in this series were obtained from the Wistar Institute of Anatomy, Philadelphia. They were received at weaning time (28 to 30 days old) and immediately upon receiving they were placed on the diets, being isolated in separate cages with open screen bottoms to prevent refection. No selection was made with respect to weight. Only male animals were used.

Table II. (Series II)

Deficient Group

Date of Determination	Oxygen Uptake per mg. water-free dry matter per 2 hrs.	Ages			Weights grams		Bald Spots	Cataract	Stomach Ulcer	Fat
		Initial Days	On Diet Days	Final Days	Initial	Final				
3/30	1.472	46	37	83	90.0	87.0	+ +	-	-	-
3/30	1.750	46	37	83	97.0	98.0	+	-	-	-
4/ 9	1.465	46	46	92	98.5	104.0	+	-	-	-
4/ 9	0.936	46	46	92	97.2	124.0	+	-	-	-
4/19	1.084	46	56	102	90.0	87.5		-	3	-
4/19	1.093	46	56	102	96.5	121.0		+	-	-
4/26	0.912	46	63	109	92.0	88.0		-	-	-
4/26	0.889	46	63	109	97.5	116.0		-	-	-
4/27	0.809	46	64	110	101.5	111.0	+	-	-	-
4/27	1.001	46	64	110	99.0	118.5		-	1	-
5/ 4	1.175	46	71	117	96.0	108.0		-	-	-
5/ 4	1.079	46	71	117	98.0	113.0		+	-	-
5/ 7	0.990	46	74	120	92.0	76.0		-	-	-
5/ 8	0.790	46	75	121	90.5	88.0		-	-	-
Normal Controls										
3/30	2.064	46	37	83	94.5	227.0				+
4/ 7	1.158	46	44	90	88.0	241.0				+
4/ 9	0.996	46	46	92	91.5	263.0				+
4/26	0.859	46	63	109	97.5	277.0				+
4/27	0.583	46	64	110	92.5	275.0				+
5/ 4	1.008	46	71	117	94.0	276.0				+

Experimental symptoms of rats on the deficient diet.

The rats of this series up to 24 days on the vitamin G deficient diet showed no striking symptoms other than sub-normal growth. After 35 days on the diet all the rats showed a generalized thinning of the hair, in a few cases tending to matting. Several showed extensive development of alopecia on the chest and legs. None of the rats were as active as the normals of the present series, and were less active than the deficient rats of Series II. In every instance the skin of the deficient rats was thinner than that of the normal rats. In a few instances the skin was extremely tender resulting in a slight hyperemia on shaving.

It is striking that, in view of the claim made by various investigators that the symptoms of vitamin G (as well as of vitamin B_1) deficiency are due in part to inanition, the oxygen uptake and the development of symptoms is not related to changes in weight.

(a) The oxygen uptake of the skin of normal and deficient rats.

In this series the skin of the deficient rats showed a lower oxygen uptake than the control rats. Reference to Fig. I (data of Table III) shows the curves for the oxygen uptake in cubic millimeters per milligram of fat-free dry matter per 2 hours plotted against days on the diet.

At no time does the oxygen uptake of the normal rats fall as low as that of the deficient rats, and at the closest approach of the two curves the normal rats are 14.3 per cent above the deficient rats, while at all other points the difference is much greater.

The oxygen uptake of the normal and deficient rats has also been calculated on the basis of the phospholipid content of the tissue used for the measurement of the oxygen uptake (i.e. as cubic millimeters of oxygen per milligram of phospholipid per 2 hours). The phospholipid in the sample of respiring tissue was calculated from a determination on a separate sample of tissue as described previously under the heading of methods of analysis, because the sample of respiring tissue was too small (50 mg.) to make a direct determination possible.

The purpose of the calculation of oxygen uptake on this basis was an attempt to provide some means of measuring the actively respiring portions of the tissues, perhaps in this way ruling out the "dilution" effect of increased connective tissue and lipids of the normal and deficient skins. As mentioned in connection with the animals of Series II it was believed possible that although the actively respiring tissue might be the same in both normal and deficient animals, the normal might have more non-respiring tissue than the deficient, thus making

comparison on the dry fat-free basis erroneous. This is in accordance with the suggestion of Bloor (7) who found that beef muscles as well as the muscles of smaller animals and birds, arranged in the order of their decreasing phospholipid contents is also the decreasing order of their physiological activity. Assuming this to be generally true it was thought that the amount of phospholipid in the respiring tissue might serve as a better index of the active cell layers than the total fat-free dry matter.

It is evident from Fig. I that the curve for the normal rats (i.e. the oxygen uptake in cu. mm. per mgm. of phospholipid in the respiring tissue) is extremely irregular, but that for the deficient rats is fairly regular. For this difference no explanation can be offered, inasmuch as the determinations were all made under the same conditions and by the same methods.

In spite of this irregularity of the plotted curve the phospholipid-oxygen uptake of the normal and deficient rats shows a much greater difference than the fat-free dry matter-oxygen uptake. Hence, if the phospholipid content can be accepted as a measure of activity, it appears that the normal skin is in a state of much higher physiological activity than the deficient rats, which is the same conclusion derived from the fat-free dry matter oxygen uptake.

On the other hand, the average values for the phospholipid content of the skin of normal and deficient rats shows some differences, as shown in the following summary:

	Phospholipid Content		
	% of of fresh skin	% of water-free dry matter	% of fat-free dry matter
Average normal	0.585	1.53	2.65
Average deficient	0.638	1.90	2.84
Difference as per cent of the normal	+9.0	+24.1	+7.1

As calculated on the three different units the per cent of phospholipid is somewhat higher in the deficient rats than in the normal. If the phospholipid content is to be accepted as a measure of the relative proportion of protoplasm, it appears from the above summary that the skin of deficient rats contains proportionally more active living matter than the skin of normal rats. If the rate of respiration depends only on this living matter, it would be expected that the respiration would be greater in the deficient rats than in the controls, if the living matter of each respired at the same absolute rate. Since the oxygen uptake per milligram of fat-free dry matter is lower in the deficient rats, it means a much greater depression of the real respiration than the figures indicate.

But, it may be that the phospholipid content of protoplasm does not remain altogether constant. It may change in vitamin deficiency. Several investigators (quoted from the review of Sinclair,(66)) have reported for vitamin B_1 deficient rats, rabbits and pigeons, a decrease of 18 to 40 per cent in the phospholipid of the brain, liver, kidney and lungs, but no change in the muscles and skin. In rats deprived of either B_1 or A the liver phospholipid is one-half to one-third of normal.

Remp and Bing (61) believe inanition to be a feature in the development of vitamin G deficiency. They conclude that in mice, both calorie deficient and vitamin G deficient, the total body phospholipid is the same (as milligrams in the total body). However, when calculated as per cent of the net body weight (calculations of P. D. A. from the data of Remp and Bing) it appears that there is actually an increase. Normal, calorie deficient and vitamin G deficient show respectively 1.10, 1.60 and 1.85 per cent phospholipid of the net body weight. The water content of the mice is not reported so it is not possible to calculate the data in other ways. The increased percentage of body phospholipid in the calorie deficient and vitamin G deficient mice may be looked upon, therefore, as a conservation of phospholipid, other constituents of the body having been called upon as sources of energy,

the phospholipid as an essential constituent of all living matter remaining unchanged and stored fat, carbohydrate and some protein being consumed.

If, as Remp and Bing conclude, the phospholipids were reduced in vitamin G deficiency the actual amount of respiring protoplasm in the defective skin would be larger than the phospholipid figures indicate, and accordingly the real rate of this protoplasmic respiration would be lower than when measured by the phospholipid content.

From these determinations it was concluded that there was a marked reduction in the rate of respiration of the skin cells of rats on a vitamin G deficient diet. To prove that vitamin G was responsible for this deficiency experiments were tried of adding flavine to the rat skin in vitro to see if it would increase the respiration.

(b) The effect of flavine in vitro on the oxygen uptake of normal and deficient rat skin.

Attempts to prepare crystalline flavines have not been successful with the quantities of material available as the source. Kuhn and co-workers were able to obtain only a few milligrams of crystalline flavine from several hundred liters of fresh whey. Booher used powdered whey as a source of flavine, but her preparation was evidently not as highly purified as that of Kuhn.

An attempt was made to prepare flavine by the

method of Booher (4). There was obtained a small quantity of red wax-like material, which when dissolved in water, was yellow with a strong green fluorescence. Even after several precipitations, and adsorption on Fuller's earth, the wax-like impurity remained. However, this material corresponded to the properties described by Kuhn, in that in water solution it was yellow with a strong green fluorescence; it was decolorized by zinc and bisulphite in acid solution, and the color returned when the fluid was decanted from the zinc powder and shaken with air; it was soluble in chloroform, and insoluble in ethyl ether.

This material was dissolved in water, and this solution was used in the preparation of Ringer-glucose solution. The final solution as used with the tissue for the determination of the oxygen uptake was still of such concentration that it was quite yellow and still retained the strong green fluorescence.

The effect of this solution on the oxygen uptake of several rats is summarized below. The values reported are in cu. mm. of oxygen per milligram of water-free dry matter:

	Without flavine	With flavine
Normal	2.03	1.88
Deficient	1.26	1.21
Normal	1.73	1.68
Deficient	1.12	1.13

These small differences are negligible because they all fall well within the experimental error of the determination of oxygen uptake, which experience has shown to be not greater than 10 per cent.

As pointed out previously, the flavine solution was not pure. This appears not to have influenced the oxygen uptake, for it is not probable that the impurities present would decrease the oxygen uptake to exactly the extent that the flavine present might increase it. However, the failure to stimulate the oxygen uptake is not surprising in view of the observations cited previously, that flavine alone does not function as vitamin G; that the flavine requires for its action as vitamin G an additional substance, the nature of which is unknown; and that flavine alone does not act as a biological oxidation catalyst in vitro without the addition of a very small amount of liver pulp to supply the supplementary substance.

The amount of material available did not permit further investigation to determine whether a supplementary substance in vitro might catalyse the oxidation of deficient rat skin more than normal rat skin. If further investigation confirms the finding that vitamin G is a complex consisting of flavine plus a supplementary substance (vitamin B_6), and that neither of these alone is sufficient to prevent pellagra, then the above observation

that flavine does not stimulate the oxygen consumption of vitamin G deficient rat skin may be interpreted to mean that in the rats described in this paper the supplementary substance was lacking, which would therefore confirm the observation of P. Gyorgi (31) previously cited, that the new fraction, vitamin B_6, is the pellagra-preventing substance.

Further investigation of this phase of the problem is to be undertaken in an effort to determine the relation of vitamin G (flavine) to the activity of the skin.

(c) Other changes in the skin of rats in vitamin G deficiency.

The percentages of water-free dry matter, of fat-free dry matter, and water are plotted against days on the diet in Fig. II. The curves for these are seen to be irregular due to the fact that they depend upon the determination of water which has been found to vary in a single animal as much as 2%, probably because of the rapidity of evaporation during the excision of the skin and the removal of the subcutaneous fat and connective tissue; and also because of unavoidable errors in the determination of the fat content, which in duplicate trials has been found to vary as much as 3%, due to variations in the water loss and to variations in the completeness of extraction.

The differences in the normal and deficient rats is great enough to indicate that in the deficient rats there is a slightly increased fat-free dry matter content, a somewhat greater decrease in water-free dry matter, and the same degree of increase in water content. Taking the averages of all the animals in the two groups, including those at the beginning which had been on the diets only a short time we have the following comparison, all as per cent of the fresh tissue:

	Water-free dry matter	Fat-free dry matter	Water
Normal	39.14	23.67	60.84
Deficient	34.15	24.42	64.13

From these figures it appears that the development of connective tissue is not impaired in the skin of deficient rats. The greatest effect is in the content of neutral fat, which is in sharp contrast to the relative constancy of the phsopholipid content.

(Insert Table III.)

(Insert Figures I and II.)

Table III.

Deficient Rats in Order of Days on Diet.

Animal Number and Control	O_2 uptake cu. mm. per 2 hrs. per mg. water-free dry matter	O_2 uptake cu. mm. per 2 hrs. per mg. fat-free dry matter	O_2 uptake cu. mm. per 2 hrs. Avg. of pairs in column 2	O_2 uptake cu. mm. per 2 hrs. per mg. phospho-lipid	O_2 uptake cu. mm. per 2 hrs. Avg. of pairs in column 4	Dry Matter H_2O-free of fresh weight %	Matter Avg. of pairs in column 5	Matter Fat-free of fresh weight %	Matter Avg. of Pairs in column 7	Water of fresh weight %	Water Avg. of pairs in column 9	Total Lipid of fresh weight %	Total Lipid of H_2O free dry matter %
23 A	3.95	9.21		135.21		30.00		11.11		70.00		17.19	57.31
23 B	3.02	4.53	6.87	96.9	116.0	29.60	28.80	19.64	15.38	70.41	70.20	9.95	33.63
22 D	3.91	6.14		129.3		31.10		19.81		68.90		11.28	36.29
22 E	3.61	5.78	5.96	124.7	127.0	30.72	30.91	18.94	19.38	69.28	69.09	11.61	38.01
21 L	2.93	4.77		122.4		36.07		22.18		63.93		13.88	38.50
21 K	2.77	5.58	5.18	106.7	114.6	35.63	35.85	17.57	19.88	64.37	64.15	18.05	50.67
14 B	1.44	2.81		103.9		38.99		19.88		61.01		19.01	49.01
14 C	1.41	2.51	2.66	90.2	97.0	40.04	39.51	22.14	21.01	59.96	60.49	17.90	44.12
15 A	1.96	2.23		115.7		30.53		26.67		69.47		3.83	12.56
15 B	1.33	1.60	1.92	83.6	99.7	34.18	32.35	28.35	27.51	65.82	67.64	5.82	17.05
9 B	1.23	1.30		72.8		32.10		30.29		67.90		1.81	5.60
9 C	1.90	2.12	1.71	112.3	92.5	32.80	32.45	29.30	29.79	67.20	67.55	3.50	10.68
7 D	1.06	1.13		53.2		30.34		28.35		69.66		1.94	6.41
7 N	1.48	1.54	1.33	106.8	80.0	32.98	31.66	30.73	29.54	67.02	68.34	2.27	6.88
11 C	1.45	1.54		92.0		31.54		29.55		68.46		1.99	6.31
11 D	1.49	2.31	1.92	82.9	87.4	34.80	33.17	22.44	25.99	65.20	66.83	12.36	35.52
2 S	.97	1.06		76.1		35.03		33.26		64.97		1.73	4.94
2 T	1.89	2.01	1.53	125.7	100.9	32.42	33.72	30.52	31.89	67.58	66.28	1.86	5.76
17 B	1.27	2.04		101.8		41.31		25.55		58.69		15.76	38.16
17 A	1.24	2.43	2.24	81.7	91.7	38.11	39.71	17.62	21.58	61.89	60.29	20.49	53.76
4 A	.736	.766		46.1		31.90		30.60		68.10		1.30	4.08
4 B	.388	.411	.59	24.2	35.1	34.34	33.12	23.56	27.08	65.66	66.88	1.45	5.82
13 B	1.03	1.43		53.40		33.07		23.81		66.93		9.26	28.01
13 C	1.29	2.18	1.80	96.7	75.0	40.52	36.79	23.89	23.85	59.48	63.20	16.63	41.05
1 A	1.36	1.59		97.9		75.21		30.08		64.79		5.12	14.56
1 B	.95	1.00	1.30	41.2	69.5	33.63	34.42	31.83	30.95	66.37	65.58	1.79	5.34
10 M	1.27	2.54		72.5		39.41		19.85		60.59		19.42	49.44
10 H	.95	1.84	2.19	43.5	58.0	38.00	38.70	16.37	18.11	62.00	61.30	15.48	48.60

Animal Number and Control	Phospholipid (P x 25.06) of fresh weight %	Phospholipid Avg. of pairs in column 1	Phospholipid of H_2O free dry matter %	Phospholipid Avg. of pairs in column 3	Phospholipid of total lipid %	Phospholipid of fat-free dry matter %	Change in weight % of initial	Total age days	Days on diet	Weight Initial gms.	Weight Final gms.
23 A	.878		2.92		5.10	6.85	-2.7	36	5	55.0	53.5
23 B	.925	.901	3.12	3.02	9.29	4.71	-2.2	36	5	67.0	65.5
22 D	.943		3.03		8.36	4.76	+2.7	37	6	55.0	56.5
22 E	.887	.915	2.90	2.96	7.67	4.69	+1.0	37	6	54.0	54.5
21 L	.870		2.41		6.26	3.92	-7.4	44	13	54.0	50.0
21 K	.934	.902	2.61	2.51	5.17	5.31	+1.7	44	13	59.0	60.0
14 B	.542		1.39		2.84	2.73	+36.0	50	22	50.0	68.0
14 C	.636	.589	1.57	1.48	3.55	2.87	+38.5	50	22	57.0	79.0
15 A	.542		1.77		14.16	2.03	+10.0	52	24	49.0	54.0
15 B	.579	.560	1.68	1.72	9.94	2.04	+7.3	52	24	54.5	58.5
9 B	.551		1.71		30.55	1.82	+15.0	63	35	63.5	73.0
9 C	.555	.553	1.69	1.70	15.85	1.89	+24.0	63	35	37.5	46.5
7 D	.626		2.06		32.22	2.20	-20.0	63	36	50.0	40.0
7 N	.472	.549	1.43	1.74	20.80	1.53	-2.0	63	36	51.0	50.0
11 C	.498		1.58		25.00	1.68	+26.0	67	39	62.0	78.0
11 D	.635	.566	1.82	1.70	5.14	2.83	+35.5	67	39	45.0	61.0
2 S	.498		1.42		28.78	1.49	+15.0	79	49	56.5	65.0
2 T	.542	.520	1.67	1.54	29.06	1.77	-1.0	79	49	49.0	48.5
17 B	.515		1.25		3.27	2.02	+45.5	79	51	57.0	83.0
17 A	.525	.520	1.38	1.32	2.56	2.98	+46.0	79	51	52.0	76.0
4 A	.506		1.58		38.85	1.65	+48.0	85	55	41.5	61.5
4 B	.410	.458	1.64	1.61	28.23	1.74	+45.0	85	55	42.0	61.0
13 B	.643		1.94		6.94	2.70	+28.0	83	55	62.5	80.0
13 C	.541	.592	1.33	1.63	3.25	2.26	+51.0	83	55	55.5	83.5
1 A	.500		1.42		9.75	1.66	+40.0	86	56	54.5	76.5
1 B	.794	.647	2.36	1.89	44.23	2.49	+37.5	86	56	40.0	55.0
10 M	.692		1.76		3.56	3.49	+83.0	106	79	44.5	81.5
10 H	.632	.662	1.98	1.87	4.08	3.86	+58.0	106	79	67.0	106.0

Table III. (continued)

Normal Rats in Order of Days on Diet.

Normal Controls	O_2 uptake cu. mm. per 2 hrs. per mg. water-free dry matter	Dry Matter per mg. fat-free dry matter	per mg. phospho-lipid	H_2O-free of fresh weight %	fat-free of fresh weight %	Water content of fresh weight %	Total Lipid of fresh weight %	Total Lipid of H_2O free dry matter %	Phospholipid (P x 25.06) of fresh weight %	of H_2O free dry matter %	of total lipid %	of fat-free dry matter %	Total age days	Days on diet	Weight Initial gms.	Weight Final gms.	Change in weight as % initial
24 C	5.00	7.20	195.5	31.47	20.86	68.53	10.60	33.71	.807	2.56	7.61	3.86	36	5	63.5	68.0	+7.1
24 F	3.73	6.58	149.3	35.18	19.89	64.82	15.28	43.44	.881	2.50	5.76	4.42	37	6	56.0	67.0	+2.0
24 H	3.99	6.46	166.6	32.93	20.75	67.07	12.17	36.96	.795	2.41	6.53	3.83	44	13	60.0	80.0	+33.0
6 M	1.94	3.83	114.5	44.87	22.74	55.13	22.12	49.31	.793	1.76	3.58	3.49	49	22	57.0	107.0	+87.7
18 A	2.26	3.99	161.7	39.31	22.23	60.69	17.08	43.45	.568	1.44	3.33	2.55	52	24	50.0	90.0	+80.0
12 A	2.06	2.85	229.7	37.53	27.05	62.47	10.48	27.92	.339	.904	3.23	1.25	63	35	59.0	109.0	+84.5
12 B	1.58	2.98	135.8	43.92	23.16	56.08	20.76	47.26	.515	1.17	2.48	2.22	67	39	44.0	104.0	+136.0
5 R	2.22	3.50	199.1	40.90	25.60	59.10	15.30	37.41	.475	1.16	1.85	3.10	79	49	43.5	113.5	+161.0
16 A	2.03	3.08	189.0	39.60	26.01	60.36	13.63	34.38	.531	1.34	3.97	2.05	79	51	58.0	128.0	+121.0
16 B	1.74	3.69	144.6	43.80	22.09	56.20	21.72	49.58	.565	1.29	2.61	2.55	80	52	45.0	115.0	+155.0
5 S	1.73	2.56	133.2	39.64	26.70	60.36	12.94	32.64	.523	1.35	4.04	1.96	85	55	42.0	122.0	+190.0
6 C	2.10	2.82	175.4	38.57	28.63	61.25	9.93	25.67	.459	1.19	4.62	1.60	86	56	43.0	123.0	+200.0
12 C	1.12	2.52	112.2	39.98	17.80	60.02	22.18	55.48	.402	1.00	1.81	2.26	89	61	46.0	136.0	+195.0
6 A	2.06	2.98	152.8	40.27	27.84	59.73	12.43	30.88	.546	1.35	4.39	1.96	105	75	58.0	158.0	+172.5
Suckling 10 days old	3.53	9.8	102.0	32.20	11.50	67.80	20.66	64.10	1.12	3.47	5.41	9.70					
Average of Normal Rats =				39.14	23.67	60.84			.585	1.53		2.65					
Average of Deficient Rats =				34.15	24.42	64.13			.638 +9.0%	1.90 +24.1		2.84 +7.1					

Figure I.

Normal and Deficient O_2 uptake in cu. mm.

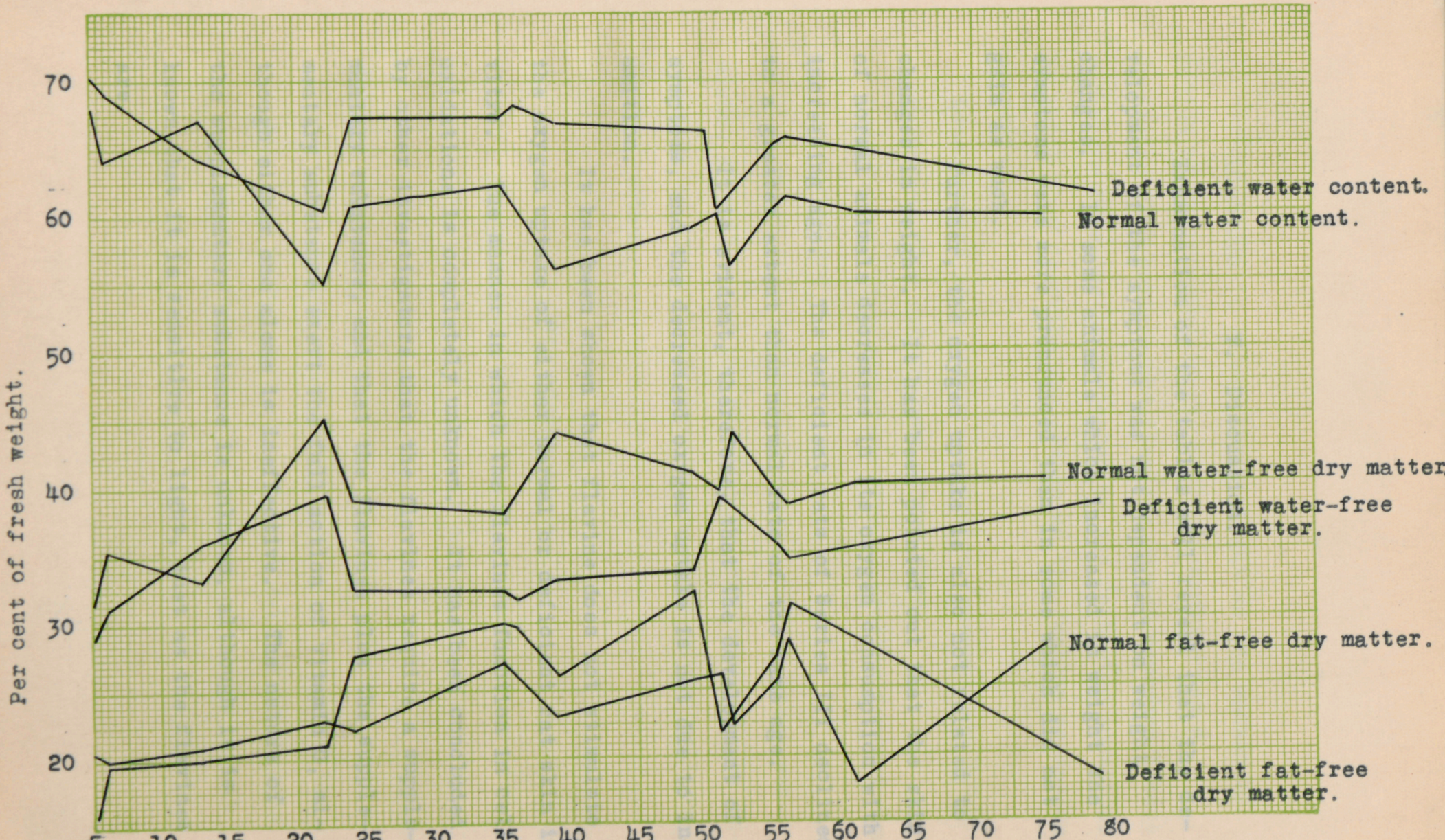


Figure II.

Composition of the Skin of Normal and Deficient Rats.

V. Discussion.

Examination of the tables will reveal that the development of the symptoms was not dependent on weight changes. In some animals which increased in weight the symptoms were more pronounced than in some which did not gain as much.

Further, the oxygen uptake is also not related to changes in weight. It has been pointed out that the skin of normal animals decreases in its oxygen consumption with increasing age. The deficient rats of Series III declined to a greater extent than normal rats of the same age.

It is evident, therefore, that the development of symptoms, and the decreased oxygen uptake is not due to inanition.

It has been shown that flavine does not stimulate the oxygen uptake of either normal or deficient rat skin in vitro. The manner in which the flavines function in cell oxidations is completely unknown. It has been concluded by other investigators that the flavines require a supplementary substance, and that the flavines plus the supplementary substance exert the full action of vitamin G, although either one alone is ineffective. The nature of the supplementary substance is unknown, although it is known that it is sensitive to light, just as the flavines are.

Although it throws little light on the manner of action of the flavine in the cell, O. Warburg and W. Christian (74) believe the flavines to act somewhat like methylene blue, as is indicated in the following equations by which they describe the action of the "yellow oxidation enzyme":

Leucoferment plus oxygen ⟶ yellow ferment plus H_2O_2.

Methylene blue can replace oxygen thus:

Leucoferment plus methylene blue ⟶ yellow ferment plus leucomethylene blue.

Until more is known about the supplementary substance as well as of the flavines it will obviously be impossible to place the flavines accurately in the cell oxidation systems.

VI. Conclusions.

(1) The oxygen consumption of normal rats declines with age, there being a sharp fall during the first few weeks to a lower level.

(2) The oxygen consumption of vitamin G deficient rats falls to a much lower level than normal rats of the same age. The difference is not related to changes in weight. The phospholipid content of the normal and deficient rat skin in relation to the oxygen

uptake indicates the same lowering of vital activity.

(3) In vitamin G deficiency the neutral fat content is markedly affected while the phospholipid content shows only a slight change.

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