

## FAT-SOLUBLE VITAMINS

### XXXVI. THE CAROTENE AND VITAMIN A CONTENT OF BUTTER\*

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As one would expect from its general dietary importance, butter has frequently been assayed biologically for its total vitamin A activity. The published results of such assays, however, vary widely, not only because of variations in butter itself, but because of variations in the method of assay, in the method of interpreting data, and in the method of expressing results. Sherman and Smith (1) for example state, "Butter appears to contain about 30 to 50 units of vitamin A per gram, ranking in richness, weight for weight, with egg yolk and fresh spinach." Fraps and Traichler (2) find similar variations and report that butter from cows on normal diets ranges from 17 to 50 Sherman units per gm. Crawford, Perry, and Zilva (3) report the minimal daily dose of summer butter necessary to maintain growth equivalent to that produced by an excess of cod liver oil at approximately 0.1 gm.

Vitamin A activity in butter fat<sup>1</sup> is due to the presence of two different chemical entities, *viz.* carotene and vitamin A. The presence of carotene was first demonstrated by Palmer and Eckles (4) who used solubility reactions, the phase test, and the position of absorption bands in the spectrum as criteria. For the presence of vitamin A itself, evidence from various sources has been available. Stephenson (5) showed that butter fat decolorized with charcoal retained its biological activity. Morton and Heilbron (6) showed the presence of an absorption band at 328  $m\mu$ , which

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<sup>1</sup> For the sake of convenience the term butter is frequently used for butter fat. Actually butter contains only 85 per cent of fat.

is the region of absorption for potent liver oil concentrates. Lundborg (7) showed that the  $\text{SbCl}_3$  reagent gives a stronger blue color with the unsaponifiable fraction of butter than can be accounted for on the basis of its carotene content. Moore (8) made a similar observation, and further identified the absorption bands at  $606 m\mu$  in the  $\text{SbCl}_3$  reaction product with butter concentrate as being due to vitamin A.

Since quantitative data of the relative carotene and vitamin A content of butter are not available, it seemed advisable that a systematic study of these relations be made. Biological methods of determination, while yielding results of great practical significance, are obviously of little value in determining the relative amounts of these active components. For this reason spectrographic methods were employed. Analyses were made on fourteen samples collected in successive months during 1932 and 1933.

The butter was made from the general supply of milk delivered at the University Dairy by neighboring farmers. It represented the composite production of various breeds of cows on rations representative of Wisconsin farm feeding practise. The butter was churned from sweet cream separated by centrifugal force, and then stored at  $4^\circ$ . Samples were collected on the 15th day of each month.

For the carotene determinations the butter was melted at  $55^\circ$  and decanted from brine and curd onto a cotton filter. The intensity of the absorption bands was measured on the melted butter fat at  $30^\circ$  by means of a Bausch and Lomb universal spectrophotometer. Readings were taken from two zero points and each sample was read at two depths, selected to give results of the greatest accuracy. For purposes of calculation the absorption values at the points of maximum absorption, *i.e.* at 460 and  $485 m\mu$ , were used, and compared with the absorption maxima of a standard solution of carotene in refined cottonseed oil.<sup>2</sup> We could have used an alternative method which involved the use of extinction coefficients for carotene recorded in the literature. However, the recorded values for the extinction coefficient of carotene absorption bands vary considerably. Duliere, Morton, and Drummond (9) report that the molecular extinction coefficient,  $E$ , of the  $462 m\mu$  band in chloroform ranges from 75,000 to 100,000.

<sup>2</sup> Wesson oil.

They use the following formula,  $E = 1/cd \log I/I_0$ , where  $E$  is the molecular extinction coefficient,  $c$  the concentration in mols per liter, and  $d$  the depth of the solution in *cm.* Later, Gillam, Heilbron, Morton, and Drummond (10) reported the value for  $E$  as 102,000. Pummerer and Rebmann (11), using a different formula, report  $\log K = 5.1$  as determined in cyclohexane. Our method of determination has the advantage that we avoided the use of these published values, which after all might not be directly applicable to carotene in solution in butter fat.

The following illustrates our method based on the 485  $m\mu$  spectral line. A solution of 0.002 mg. of carotene<sup>3</sup> per cc. of cottonseed oil at a depth of 1 cm. gave a value for  $\log I/I_0$  of 0.44. July butter at a depth of 0.5 cm. gave a value of 0.86 equal to 1.72 for a 1 cm. depth. Checked at 0.75 cm. it gave a value of 1.28 equal to 1.71 for 1 cm. The value 1.72 was therefore accepted as approximately correct. Solving for  $x$  in the proportion  $1.72 : 0.44 :: x : 0.002$ ,  $x$  we found was equal to 0.0078 mg. of carotene per cc. or 0.0086 mg. of carotene per gm. of butter fat.

When the 460  $m\mu$  line was used these values were duplicated.  $\log I/I_0$  for the carotene solution at 1 cm. equaled 0.53 and for July butter 1.05 at 0.5 cm. and 1.50 at 0.75 cm., which are equal respectively to 2.10 and 2.00 for 1 cm. depth. Accepting the average, *viz.* 2.05, and solving for  $x$  in the proportion  $2.05 : 0.53 :: x : 0.002$ , we found  $x$  gave a value of 0.0078 mg. of carotene per cc. or 0.0086 mg. of carotene per gm. of butter.

The accuracy of this method of determining carotene was checked by adding carotene to February butter, as an example of a butter of minimal carotene content, and to a sample of uncolored oleomargarine in such amounts as were calculated to give these samples the color intensity of June butter. As an analysis of February butter showed that it contained 0.0022 mg. of carotene per cc. and June butter 0.0062 mg. of carotene per cc., 0.0040 mg. of carotene was added to each cc. of February butter. 0.0062 mg. of carotene was added to each cc. of oleomargarine. After these additions actual determination of the total pigmentation

<sup>3</sup> The carotene used was apparently pure  $\beta$ -carotene as it showed no optical activity and melted at 181°. It was part of a preparation sent by this laboratory to the British Medical Research Council for use in the preparation of the international standard for vitamin A.

by the spectrophotometer showed that the colored February butter now contained 0.0060 mg. of carotene per cc. and the colored oleomargarine 0.0062 mg. of carotene per cc.

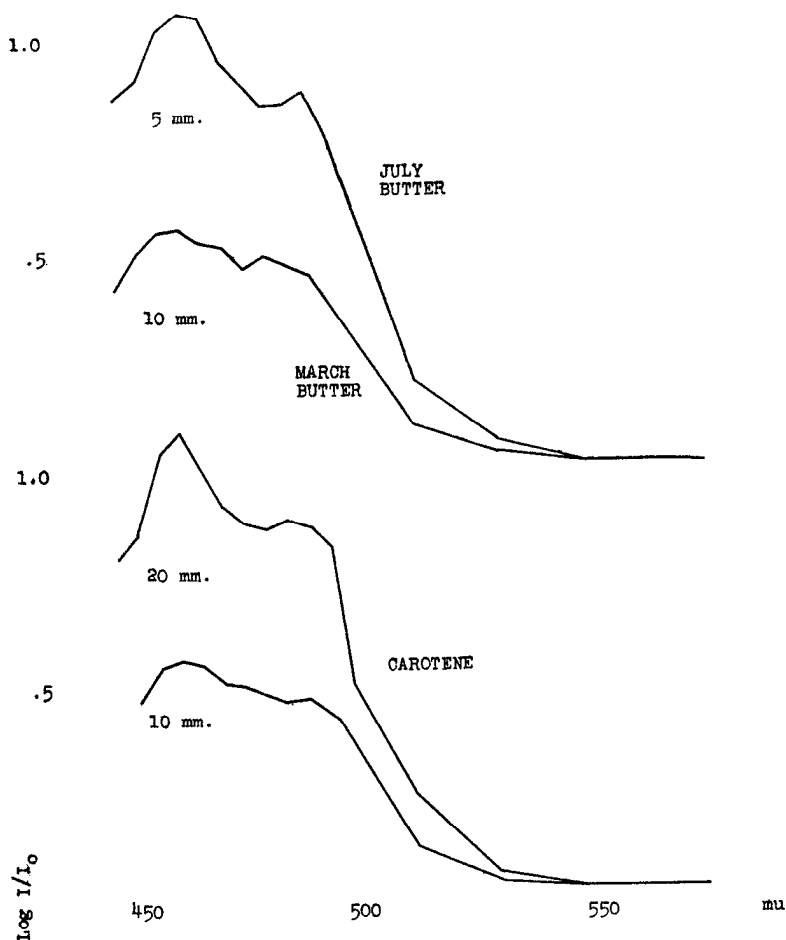


FIG. 1. The spectral absorption curves in the visible part of the spectrum obtained from butter fat of high and low carotene content (July and March respectively). The observations were made with a Bausch and Lomb spectrophotometer with the butter fat at depths of 5 and 10 mm. so as to facilitate direct comparison with a solution of carotene in cottonseed oil which contained 0.002 mg. in each cc. The similarity in the shape of the curves is considered significant.

That natural butter pigment consists for the most part of carotene was demonstrated as follows: 40 gm. of August butter were saponified by boiling with 12 per cent alcoholic KOH. The mixture was diluted with water and then extracted with ether. The ether was evaporated. The residue was taken up in petroleum ether. This was washed three times with an equal volume of 92 per cent methyl alcohol. It was found that 95 per cent of the pigment remained in solution in the petroleum ether. The absorption curve of the petroleum ether solution, as obtained in the spectrophotometer, was found to be identical with that of carotene (Fig. 1).

*Determination of Vitamin A*—Vitamin A was determined in the butter by photographing the intensity of the 328  $m\mu$  band of the unsaponifiable matter dissolved in methyl alcohol. Before so doing it was necessary to purify the unsaponifiable matter in order to remove so far as possible substances other than vitamin A which absorbed in the same region.

The unsaponifiable fraction was prepared as follows: 15 gm. of butter were refluxed in a stream of nitrogen for 30 minutes with 125 cc. of aldehyde-free freshly prepared 12 per cent alcoholic KOH. 125 cc. of water were added, and the mixture was cooled to 4°. 150 cc. of ether were then added to the cold solution followed by an addition of 500 cc. of cold water. The ether was drawn off and the aqueous alcoholic fraction was then extracted three successive times with additional portions of ether of 50 cc. each. The combined ether solutions were washed with water repeatedly, dried over  $\text{Na}_2\text{SO}_4$ , and freed from ether with nitrogen under reduced pressure. The residue was dissolved in 15 cc. of hot methyl alcohol and the impurities were crystallized out by cooling several hours to  $-72^\circ$  in a mixture of solid  $\text{CO}_2$  and acetone. The cold solutions were filtered, washed with cold, methyl alcohol, and made up to such a volume that the photographed solution showed a value at 328  $m\mu$  and a depth of 2 cm. for  $\log I/I_0$  of approximately 1.0.

Absorption spectra were photographed through a Hilger quartz spectrograph equipped with a sector photometer and a hydrogen discharge tube. Since pure vitamin A as a standard was not available, calculations of the vitamin content of the butter were made with a value for the extinction coefficient of this vitamin published by Heilbron and coworkers (12).

The following illustrates the method of calculation: A concentrate of June butter made according to the above procedure and containing the equivalent of 0.166 gm. of butter per cc. showed a value for  $\log I/I_0$  of 0.8 when photographed through a 2 cm. cell (Fig. 2). The value for the extinction coefficient of 1 gm. of butter at  $328 m\mu$  was therefore equal to  $(0.8/2) \div 0.166$ , when 2

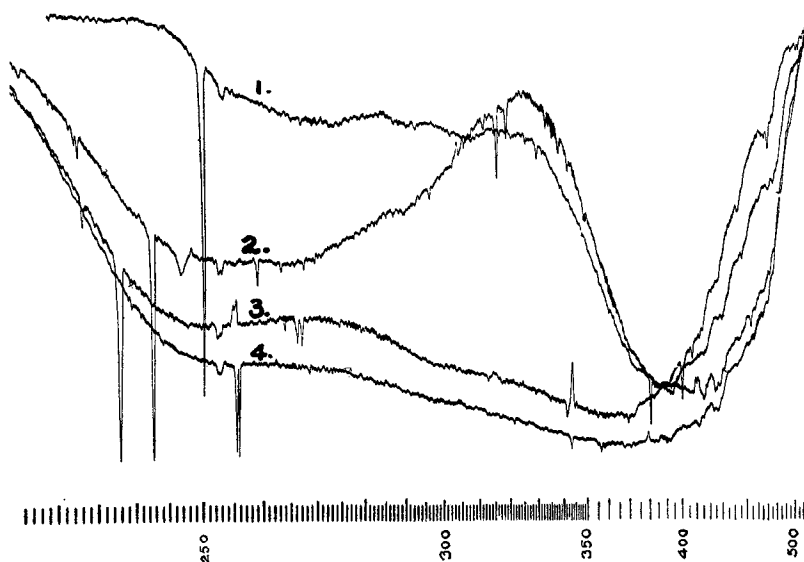


FIG. 2. Spectral absorption curves: Curve 1, a concentrate of unsaponifiable constituents from butter fat dissolved in methyl alcohol where 1 cc. = 0.166 gm. of June butter; Curve 2, a vitamin A concentrate from halibut liver oil diluted to 1:1,600,000 in methyl alcohol; Curve 3, carotene in methyl alcohol; and Curve 4, methyl alcohol. The butter concentrate shows absorption both at  $450 m\mu$  and at  $325 m\mu$ . This is accounted for on the basis of its content of both carotene and of vitamin A. Absorption at wave-lengths shorter than  $300 m\mu$  is presumably due to the presence of other substances in the butter concentrate.

The vitamin A concentrate from halibut liver oil (No. 210946) was obtained from the Abbott Laboratories, Chicago. Their biological assay indicated 1,600,000 u.s.p. units of vitamin A per gm. The antimony trichloride test showed the presence of 1,500,000 blue units per gm. measured at 3B in a Lovibond tintometer, or 3000 times as many as in standard cod liver oil. The fact that the extinction coefficient  $E$  (1 per cent concentration at a depth of 1 cm.) equaled 750 indicates that it contained approximately 60 per cent vitamin A by weight.

equals the depth of the cell in cm. and 0.166 equals the gm. of butter per cc. of the solution photographed. Therefore,  $E$  (1 gm. at a depth of 1 cm.) = 2.4 at 328  $m\mu$ .

The vitamin A values were calculated according to the Beer-Lambert law,  $E = 1/cd \log I/I_0$ , where  $E$  is the extinction coefficient,  $d$  the depth of the cell in cm., and  $c$  the concentration. If the extinction coefficient for pure vitamin A be taken as equal to 1300 (12)<sup>4</sup> and substituted in the above formula, then for June butter  $c = 0.000308$  per cent or 3.08 $\gamma$  vitamin A per cc. of solution. 0.166 gm. of butter = 3.08 $\gamma$  vitamin A. 1 gm. of June butter = 18.6 $\gamma$  vitamin A.

Several samples of each butter were analyzed by the above method and the values were averaged. That there was no loss of vitamin A due to storage was shown by the fact that preparations made from the same butter at intervals of 6 months showed the same absorption. The stability of the purified vitamin solution in methyl alcohol at 0° was likewise demonstrated.

The fact that our manipulative procedure did not result in any loss of vitamin was demonstrated as follows: A solution of April butter treated as above described showed  $\log I/I_0$  (at a depth of 2 cm.) equal to 0.83 at 328  $m\mu$  when 1 cc. contained 0.333 gm. of butter. 30 cc. of this solution, which were equivalent to 10 gm., were added to 10 gm. of April butter, and the mixture was put through the process of saponification and purification outlined above. It was made up to 60 cc. 1 cc. therefore equaled 0.166 gm. of butter plus 0.166 gm. of butter equivalent of concentrate. Any loss due to manipulation would have been reflected in a decreased absorption, since one-half of the active material had been put through two procedures, while the other half had only been put through one. However,  $\log I/I_0$  (at a depth of 2 cm.) of this solution equaled 0.83, the same as the original solution.

Some of the samples prepared showed the presence of a substance which absorbed strongly in the region of the spectrum below 300  $m\mu$ , and this substance was present in sufficient amount to absorb some of the light in the region between 320 and 330  $m\mu$  as well. The observed values for  $\log I/I_0$  at these wave-lengths

<sup>4</sup> A recent note by Carr and Jewell (13) states that the extinction coefficient of pure vitamin A is 1600. The use of this constant would lower our calculated values for vitamin A by 18 per cent.

were therefore not accurately indicative of the vitamin A content of these samples.

Substances found in butter and known to absorb in this region include carotenoid pigments, sterols, and unsaturated fatty acids. In the solutions as photographed, however, there was not enough carotene present to exert an appreciable effect upon the 328  $m\mu$  band (Fig. 1). The effect of sterols was determined by cooling saturated methyl alcohol solutions of cholesterol, ergosterol, and of irradiated ergosterol to  $-72^\circ$  in a mixture of solid  $\text{CO}_2$  and acetone as carried out with the unsaponifiable fraction of the butter fat samples. The filtered solutions were diluted with 2 volumes of methyl alcohol, bringing them to approximately the same concentration as the photographed butter solutions, if the butter solutions were saturated with sterol at  $-72^\circ$ . Analysis of these sterol solutions showed that they did not absorb at 320 to 330  $m\mu$ . The effect of fatty acids or of glycerides—possibly present in traces—was tested by resaponifying the butter concentrates and purifying them as before, since free fatty acids are known to absorb at 320  $m\mu$  (14). In no case was the absorption in the shorter ultra-violet region decreased. When 1 per cent KOH was added to the methyl alcohol solution of the purified butter concentrate, absorption in the shorter ultra-violet region increased markedly, and absorption in the 328  $m\mu$  region also increased. While alkali itself does not absorb in this region, it appeared possible that the increased absorption might be due to the effect of resins produced by the action of alkali upon aldehydes. Such aldehydes could come not only from the alcohol used in the saponification—a possibility which was minimized by using alcohol of suitable purity—but also from the oxidation and hydrolysis of unsaturated fatty acids in the butter fat. Resins produced from old alcohol showed strong absorption in the ultra-violet region.

Among methods designed to determine whether interfering agents in the butter concentrates absorb at 328  $m\mu$ , an obvious procedure would have been to remove carotene and vitamin A from the solution before analyzing the residue spectroscopically. However, the very fact that the interfering agents followed the vitamin through the purification process argued for some similarity between them and the vitamin, and procedures used to destroy carotene or vitamin A would probably, therefore, have



altered the interfering agent as well. Nevertheless, such attempts to remove vitamin A were made. Methyl alcohol solutions of vitamin A concentrates prepared from butter and halibut liver oil were transferred to ether and excessively exposed to light. They were first irradiated in air for 4 hours under a Sunshine carbon arc followed by 4 hours exposure to the rays of a cold quartz lamp. During these exposures a Pyrex filter which removed radiations below  $325\text{ m}\mu$  was used to prevent the destruction of

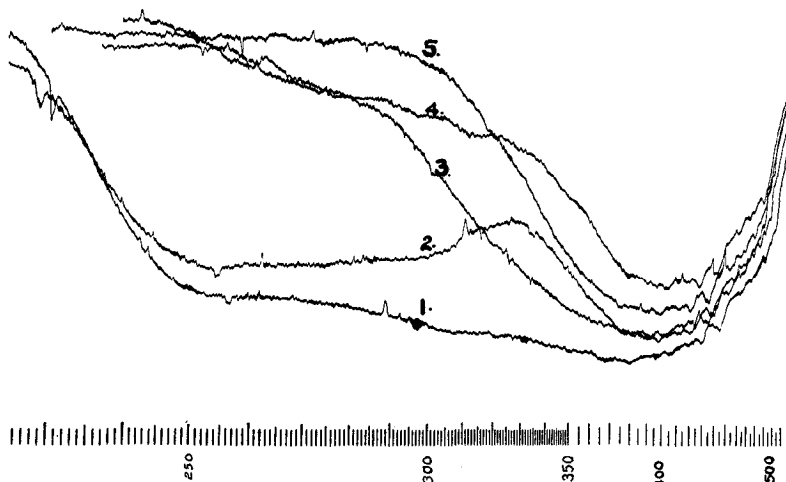


FIG. 3. Spectral absorption curves: Curve 1, methyl alcohol; Curve 2, a vitamin A concentrate (the same as in Fig. 2) from halibut oil diluted 1:3,200,000 with methyl alcohol; Curve 3, the same solution oxidized; Curve 4, a concentrate of unsaponifiable constituents from butter fat (1 cc. = 0.2 gm. of August butter); Curve 5, the same solution oxidized. Oxidation of both the butter and the halibut concentrates resulted in decreased absorption at  $325\text{ m}\mu$  and a very marked increase in absorption below  $300\text{ m}\mu$ .

those agents which absorb in this region. The ethereal solutions were then transferred to Pyrex tubes and exposed to light from a window of northern exposure for 3 days. Spectroscopic examination of these solutions showed very little absorption in either the  $325\text{ m}\mu$  region or in the shorter ultra-violet region.

An attempt was also made to destroy selectively vitamin A and carotene in butter concentrates by oxidation. The concentrates were transferred to toluene, and air was bubbled through them

for 3 days at room temperature. After evaporation of the solvent, the residues were dissolved in methyl alcohol. Spectroscopic analysis showed that oxidation of a vitamin A concentrate from halibut liver oil resulted in a disappearance of the  $328\text{ }m\mu$  band and the appearance of general absorption in the shorter ultra-violet region. The new band showed a value for  $\log I/I_0$  at  $280\text{ }m\mu$  many times larger than that of  $\log I/I_0$  at  $328\text{ }m\mu$  for an unoxidized solution of the same concentration. While the oxidized halibut liver oil concentrate showed some absorption at  $328\text{ }m\mu$  this absorption was much less than that observed at shorter wavelengths (Fig. 3). Oxidation of the butter concentrates resulted in a marked increase in absorption in the shorter ultra-violet region, and such oxidized concentrates also absorbed at  $325\text{ }m\mu$ . On dilution, however, this absorption decreased. It therefore appears that the interfering agents in the original butter concentrates are oxidation products of carotenoid substances and that these substances do not appreciably absorb light at  $325\text{ }m\mu$ , when their concentration as measured by the absorption at  $280\text{ }m\mu$  is relatively low.

Since the absorption curves of the butter concentrates all showed the presence of substances in addition to carotene and vitamin A, as evinced by absorption below  $300\text{ }m\mu$ , a minimum effect of these unknown substances upon the  $328\text{ }m\mu$  band was insured by arbitrarily discarding all analyses in which the absorption at  $280\text{ }m\mu$  exceeded that at  $328\text{ }m\mu$ .

Parenthetically it may be stated that in connection with another research in progress at this laboratory, samples of butter made from irradiated milk were analyzed for their content of carotene and of vitamin A. No loss of either component was observed although the milk had been exposed to ultra-violet rays sufficiently to have imparted to it a potency of 50 Steenbock units of vitamin D per quart. The acidity of the cream at churning time was likewise without effect. Butter made from neutral cream contained the same amount of carotene and vitamin A as that made from cream with an acidity of 0.42 per cent lactic acid.

The results of our carotene and vitamin A determinations are shown collectively in Table I. The carotene values ranged from a minimum of  $2.0\gamma$  per gm. of butter fat in April to a maximum of  $8.6\gamma$  per gm. in July. Vitamin A values ranged from  $9\gamma$  in April

to 20 $\gamma$  in August. A general parallelism was found between the carotene and the vitamin A content of the samples. However, the July butter presented an anomaly, being lower in vitamin A content than either the June or August samples. We are unable to explain this. The error did not arise through storage of the butter since the irradiated July butter was also lower in vitamin A content than irradiated June and August butter. It, however, is

TABLE I  
*Seasonal Variation in Carotene and in Vitamin A Content of Butter*

Date	Carotene per gm. butter fat	$E_{1\text{ cm.}}^{1\text{ gm. butter}}$ at 325 $m\mu$	Vitamin A per gm. butter fat*
	$\gamma$		$\gamma$
Feb., 1932.....	2.2	1.4	11
Mar., 1932.....	2.2	1.1	9
Apr., 1932.....	2.0	1.1	9
May, 1932.....	2.4	1.7	14
June, 1932.....	7.1	2.4	19
July, 1932.....	8.6	1.9	15
Aug., 1932.....	5.8	2.5	20
Sept., 1932.....	5.2	2.4	19
Oct., 1932.....	4.5	2.1	17
Nov., 1932.....	4.0	2.0	16
Dec., 1932.....	3.0	1.9	15
Jan., 1933.....	2.4	1.5	12
Feb., 1933.....	2.2	1.5	12
Mar., 1933.....	2.0	1.3	10

\* The values for vitamin A are calculated on the assumption that the absorption at 325  $m\mu$  is due entirely to vitamin A. Bills has recently shown that when this assumption is applied to liver oils, the maximum variation between biological and spectrographic results is 20 per cent; and the average is less than 10 per cent (15).

possible that the cream from which this butter was made had been partly collected from another source. We had no means of checking this up.

In order to calculate the relative importance of carotene and vitamin A in the extent to which they contribute to the vitamin A activity of butter, we may tentatively accept Sherman's figure of 50 Sherman rat units per gm. as the biological value of our summer butter. Preliminary observations indicate that 1 Sherman unit

is equal approximately to 1.3 international units—1 international unit being the activity of  $1\gamma$  of standard carotene. Sherman's value for summer butter then becomes 65 international units of which, according to our determinations, no more than 8 units are due to carotene.

Another method of calculation involves the acceptance of vitamin A values. Euler and Karrer (16) and Drummond (12) report that for a rat the daily minimum physiological dose of pure vitamin A which is capable of inducing growth lies between 0.1 and  $0.5\gamma$ . With the average value 0.3 taken as a basis for our calculations  $20\gamma$  of vitamin A in summer butter therefore equal 66 biological units or 85 international units of vitamin A.  $8\gamma$  of carotene equal 8 international units of carotene in the same butter.

The two methods of calculation are not in exact agreement, the reason for which, in view of the assumptions made, can be easily understood. Apparently the main difficulty in securing better agreement at present lies in the large variation in the response of different animals to a uniform dosage of active material. It, however, appears that less than 15 per cent of the biological activity in butter is due to carotene. From a practical point of view the important difference in the biological potency of summer and winter butter is due to its content of vitamin A as such. The use of carotene as an artificial butter color would not materially increase the biological potency of the winter butter unless enough pigment were added to color the butter a deep red.

The variations in the carotene and the vitamin A content of butter are of some physiological interest since the carotene content varies 400 per cent, whereas the vitamin A content varies only 200 per cent. This is not a surprising result when the diet contains an excess of carotene, for carotene is obtained directly from the diet and would be expected to vary more widely than vitamin A, as the latter is a product of animal metabolism. The well known capacity of the liver to store excess vitamin A could easily account for the greater constancy of this component in the butter fat. Copeland and Fraps (17) and Moore (8) have calculated the relation between the vitamin A activity of summer butter and the vitamin A activity of a cow's diet when on pasture. Both workers find an enormous excess of dietary vitamin A. It appears that the problem of increasing the vitamin A content of summer butter

by means of dietary adjustment is not promising because the animal is already well supplied with carotene in proportion to its ability to convert it into vitamin A. On the other hand, the problem of adjusting the winter diet of the cow so as to maintain the summer level of vitamin A in milk and butter fat is one worthy of consideration.

#### SUMMARY

Spectrographic methods of analysis, which detect small variations in the occurrence of substances showing selective spectral absorption revealed that the vitamin A activity of butter can be accounted for on the basis of its content of carotene plus vitamin A. In the sum total of biological activity carotene is not of major importance, since it accounted for only 15 per cent of the total. Quantitatively the amount of carotene ranged from  $2.0\gamma$  per gm. of April butter to  $8.6\gamma$  per gm. of July butter. Vitamin A values ranged from  $9\gamma$  per gm. of April butter to  $20\gamma$  per gm. of June butter. These variations were seasonal and regular. No loss of either active component was observed as the result of storage for 6 months at  $0^\circ$ , or in consequence of ultra-violet irradiation of the milk from which the butter was made. Carotene additions to winter butter in an attempt to increase the vitamin A activity up to that of summer butter do not appear to be practical unless the public should be found to be willing to accept a much more highly colored product than it does at present.

We are indebted to Professor H. C. Jackson and to Mr. K. G. Weckel of our Dairy Department for the preparation of the butter samples. To them we wish to express our appreciation.

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