CLX. A STUDY OF SEASONAL VARIATION IN BUTTER-FAT.

I. SEASONAL VARIATIONS IN CAROTENE, VITAMIN A AND THE ANTIMONY TRICHLORIDE REACTION.

By ROLAND GORDON BOOTH, STANISŁAW KAZIMIERZ KON
(*from the National Institute for Research in Dairying, University of Reading*),

WILLIAM JOHN DANN AND THOMAS MOORE
(*from the Nutritional Laboratory, University of Cambridge
and Medical Research Council*).

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IN January 1932 work was started in the National Institute for Research in Dairying to investigate the variations in the vitamin content of typical English milk as determined by the season of the year and the nutritional condition of the cow. This work, which was planned to cover two complete years, and which will be described in detail later, is mainly concerned with the accumulation of data obtained in routine biological tests carried out according to accepted methods. In several instances however unexpected difficulties have been encountered, and it has been necessary to seek new explanations of the observed facts. Among other occasions this was the case when it was attempted to apply the well-known colorimetric and spectroscopic methods to the estimation of carotene and vitamin A in butter-fats, and the present joint work with the Nutritional Laboratory is concerned with our attempts to overcome this difficulty.

In assaying the vitamin A of cod-liver oils by the antimony trichloride method it is well known that the blue value calculated per unit of the original oil may be greatly increased as the result of saponification. Green [1932] and Moore [1932] have already noticed that the blue value given by butter-fat is similarly increased on saponification, and this finding has been confirmed in the National Institute for Research in Dairying in routine tests on over 150 samples of butter-fat. What specially attracted our interest however was the fact that the blue value was not always increased in the same ratio, and that in general the increase was much greater in samples of butter obtained from cows at pasture ("summer butters") than in samples obtained from stall-fed cows receiving diets totally or partially deficient in green fodder ("winter butters"). In addition it was noticed that the colours produced by the untreated winter and summer butter-fats differed qualitatively. The maximum intensity of blue in the case of winter butter was given immediately on the addition of the antimony trichloride reagent, after which fading took place very rapidly (2-5 secs.) without the formation of any intermediate tint. With summer butters on the other hand the blue colour was usually more stable, sometimes taking a few seconds to reach its maximum value, while fading was preceded by the change in the colour of the solution from the original greenish-blue to a dull reddish-blue. Now it is well known that the blue colour given by vitamin A in the antimony trichloride test is "inhibited," a term which may be considered to embrace diminution in intensity, alteration in tint or acceleration in the

rate of fading, by a wide variety of substances [Corbet *et al.*, 1933]¹. Thus unsaturated acids and oils cause inhibition when present in relatively high concentration [Norris and Church, 1930], while substances of the indole type [Emmerie *et al.*, 1931] and also a highly unsaturated fraction obtained from the acids derived from cod-liver oil [Emmerie, 1933] appear to act more specifically and cause inhibition when present in very small amounts. In a preliminary communication [Booth *et al.*, 1933] we have already suggested that the essential cause of the different behaviour of untreated summer and winter butter-fats in the antimony trichloride reaction lies in the fact that not only the vitamin A content, but also the inhibitory power of the fat is increased in summer butters. We have also pointed out that the free fatty acids prepared from summer butter differ from those from winter butter in showing much stronger absorption at 230 $m\mu$, and much more rapid brown colour formation when treated with the antimony trichloride reagent. Since however it is not yet clear whether these two properties are genuinely related to the increased inhibitory power of summer butter-fat we shall not include data obtained on the free fatty acids in the present paper but shall confine ourselves to a description of inhibitory power only in so far as it affects the colorimetric estimation of the carotene and vitamin A.

EXPERIMENTAL.

The 15 butter-fats examined in the present work were selected from a large collection of samples which had been accumulated and examined in the course of about 13 months as the result of bi-weekly collections of milk from the typical herd of Shorthorn cows kept at Shinfield. The butter was churned in the Institute's experimental dairy and was unsalted. It was rendered without delay. When not in use the butter-fats were stored in a refrigerator. No noticeable deterioration occurred under these conditions.

Carotene estimations. There is little evidence that any pigment other than β -carotene is normally present in significant amounts in butter-fat, and in the present work the intensity of yellow pigmentation has been taken as a measure of the carotene concentration. The yellow units per g. of butter-fat were measured in a Lovibond tintometer using a cell of such thickness as to give a reading of 2–5 Y. This reading was then corrected for thickness of the cell and for the difference of the specific gravity of butter-fat from unity^{2,3}.

¹ Interference in the application of the $SbCl_3$ reaction may also arise through clouding of the reagent (as by water, alkali, medicinal paraffin or squalene), or through the development of an intense colour by the substance added which masks the vitamin A blue (as in the case of the brown colour formed by large amounts of unsaturated acids, probably through impurity, or by rancid fats). These forms of interference can hardly be considered to represent true "inhibition."

² The accuracy of observation is limited by two factors. On the one hand the matching of shades of more than 10 Y is very difficult, involving a great personal variation according to the amount of red introduced. On the other it is found that the readings obtained on using cells of different thickness (1 cm., $\frac{1}{2}$ cm., $\frac{1}{4}$ cm.) do not show a linear relationship. The same effect is observed when the thickness of the cell is kept constant, and the fat diluted with a suitable solvent. The plan of keeping the reading within defined limits, as described above, seemed to be least objectionable and was always adopted except in early experiments carried out before a $\frac{1}{4}$ cm. cell had been acquired. In these cases readings of the order of 10 Y, taken in a $\frac{1}{4}$ cm. cell, were subsequently corrected to allow for the slightly lower reading that would have been obtained if a $\frac{1}{4}$ cm. cell had been used.

³ In earlier experiments yellow values were estimated both on the untreated butter-fat and on the non-saponifiable residue. The values obtained in the latter case, however, always indicated a quantitative transference of pigmentation to the non-saponifiable residue, and observations on the latter therefore appeared to be unnecessary. In the present paper data obtained using the untreated butter-fat only are given.

Example. Sample in $\frac{1}{2}$ cm. cell gave reading of 3.2 Y.

$$\text{Y.U. per g.} = 3.2 \times 2 \times \frac{1}{0.9} = 7.1.$$

Blue colour estimation. Untreated butter-fat. The blue values of the untreated butter-fats were estimated by treating 0.5 g. of the melted fat with 2 cc. of the SbCl_3 reagent in a 1 cm. cell, and observing the maximum blue reading attained. Since the colours given were extremely transient, especially in the case of winter butters, observations could only be carried out by setting the tintometer at the expected reading as indicated by preliminary trials, and then, after rapidly adding the reagent to the fat, noticing whether the maximum colour produced was more or less than the expected reading. Through practice fairly consistent results could be obtained in this way.

Blue colour estimation. Non-saponifiable residue. Saponification of the butter-fats was effected by boiling together 5 g. of the fat, 2.2 cc. of a saturated solution of KOH in distilled water and 10 cc. of ethyl alcohol. In earlier experiments boiling was continued for 1 hour under a reflux condenser, but this procedure was subsequently modified since saponification was found to be complete after boiling for 2 minutes. The alteration in the time of boiling had no effect on the blue value of the non-saponifiable residue, and it will therefore be unnecessary to indicate which procedure was adopted in individual samples. To obtain the non-saponifiable residue the soaps were diluted with 40 cc. of water and extracted with 3 portions of 50, 25 and 25 cc. of ether. The extracts were combined, washed with water, dried with anhydrous sodium sulphate and evaporated on a water-bath, the last traces of solvent being removed under diminished pressure.

The non-saponifiable residue so obtained was dissolved in 5 or 10 cc. of chloroform. An amount of this solution expected to give a blue reading of 3-5 was made up to 0.5 cc. with chloroform and treated in a 1 cm. cell with 2 cc. of the antimony trichloride reagent. The colour produced did not fade so rapidly as in the case of the untreated butter-fats, but to obtain maximum values it was necessary in some cases to take readings rapidly. Results were calculated as follows.

Examples. Untreated butter.

0.5 g. with 2 cc. SbCl_3 reagent gave 2 B 0.5 Y.

$$\text{B.U. per g.} = 2 \times 2.5 \text{ (total vol. of mixed solutions)} \times \frac{1}{0.5}$$

$$= 10 \text{ B.U. per g.}$$

Non-saponifiable residue from 5 g. butter-fat was dissolved in 10 cc. CHCl_3 . 0.3 cc. of this solution with 0.2 cc. CHCl_3 + 2 cc. SbCl_3 gave 4 B 1 Y.

$$\text{B.U. per g. butter-fat} = \frac{4 \times 2.5 \times 10}{5 \times 0.3}$$

$$= 67.$$

The above colorimetric methods, of course, do not permit a high degree of accuracy. Personal errors in matching the colours are difficult to exclude, particularly when a high proportion of a second colour (*e.g.* yellow in the case of the SbCl_3 blue) or the use of neutral glasses is necessary to obtain the correct shade. Moreover the conditions of illumination of the tintometer, which in the present instance were quite different at Shinfield (artificial light) and Cambridge (diffuse reflected daylight), must be taken into account. To avoid variations arising from errors of the above type the results obtained in the two laboratories were not intermixed, but each series was taken from results obtained in one laboratory. In most cases the general conclusions reached were confirmed by parallel work in the other laboratory.

Table I.

Date	Yellow value (Y.U. per g.) Untreated butter-fat	Blue value (B.U. per g.)		Ratio blue value N.S.R. blue value fat
		Untreated butter-fat	Non- saponifiable matter	
1932 April 26	2.4	8	16	2
Cows went out to pasture April 26th.				
May 3-4	10.5	10.5	66	6
May 10-16	17.5	7	66	9
June 22-28	23	10	63	6
July 12-Aug. 2	17.5	7	62	9
Sept. 30-Oct. 13	19.4	8	67	8
Oct. 31-Nov. 3	16.5	7	72	10
1933 Jan. 26-30	2.6	7	42	6
Feb. 24-27	2.8	12.5	35	3
March 24-27	3.0	13	40	3
April 14-17	2.3	12	34	3
April 19-20	3.2	13	36	3
Cows went out to pasture April 20th.				
April 27-28	7.2	9	64	7
May 9-11	13	8	68	8.5
May 19-22	18	7.5	72	10

Fig. 1. Seasonal variations in butter-fat. The upper continuous curve indicates the SbCl_3 blue value determined on the non-saponifiable residue, the lower continuous curve the blue value as determined on the untreated butter-fat. The broken curve indicates carotene expressed as yellow units determined on the untreated fat. The blue and yellow scales have been adjusted on the assumption that 2 Y.U. of carotene are equivalent biologically to about 5 B.U. of vitamin A.

Seasonal variation in carotene content. The seasonal variation in carotene content as indicated by the yellow value is given in Table I and shown graphically in Fig. 1. The first value given relates to a very faintly coloured butter-fat obtained during the last weeks of stall feeding in April 1932. In agreement with

well established precedent there was a sharp rise in the carotene content coinciding with the turning out of the cows to pasture, after which the value remained almost constant until the autumn, when a gradual fall began which continued throughout the winter, during which, of course, stall feeding was adopted with an increasing scarcity of green fodder. The butters of March and April 1933 were again very faintly coloured, and the rise in carotene content was repeated on turning out the cows to pasture as in the previous year.

Seasonal variation in the blue value of the untreated butter-fat. Data are given in Table I and Fig. 1. The value varied between comparatively narrow limits (7–13 B.U. per g.) and was usually somewhat greater in "winter" than in "summer" butter-fats.

Seasonal variation in the blue value of the non-saponifiable residue (see Table I and Fig. 1). In contrast with the case of the untreated butter-fat, the blue value of the non-saponifiable residue showed a marked seasonal variation. The value rose sharply when the cows were turned out to pasture in the spring of 1932, remained constant during the summer and autumn, declined during the winter and rose again when the cows were turned out to pasture at the end of April 1933. The variation thus followed the carotene content, although the disparity between the winter minimum and summer maximum was not so great.

Seasonal variation in the ratio of the blue values as determined on the non-saponifiable residue and on the untreated butter-fat. In the above experiments the blue value per g. of butter-fat was invariably greater when the determination was carried out on the non-saponifiable residue than when the butter-fat was treated directly with the SbCl_3 reagent. It will be seen from Table I, however, that the ratio of the two values was not constant, but was generally greater in summer than in winter samples.

Seasonal variation in the inhibitory power of the butter-fat in the SbCl_3 reaction of vitamin A. The inhibitory powers of the winter and summer butter-fats were compared by observing the reduction in blue reading caused by the addition of a fixed amount of the butter-fat to a fixed amount of vitamin A concentrate (B.D.H.) before treatment with the SbCl_3 reagent. It must be emphasised that both winter and summer butter-fats interfered in the SbCl_3 reaction of vitamin A, although in different ways. With winter butter-fat the first flash of colour produced approximated to that due to the added concentrate, after which rapid fading took place. With summer butter, on the other hand, the first flash of colour was completely inhibited, and instead the much less intense dull blue which appears to be given by the vitamin in the presence of inhibitory substances gradually developed. By taking readings immediately after the addition of the reagent, therefore, a decided seasonal difference could be demonstrated.

In Table II data are given showing the effect of adding 0.4 cc. of butter to 0.0055 mg. of a vitamin A concentrate, which by itself gave a reading of 7 B.U. when dissolved in 0.5 cc. of chloroform and treated with 2 cc. of the SbCl_3 reagent. The colour readings observed immediately after adding the reagent and also after standing for 30 seconds are given. In Fig. 2 these results are shown graphically. The inhibitory power shows a seasonal variation similar to that already shown to occur in the carotene and vitamin A contents¹.

¹ In the above experiments the blue colour observed must have been derived not only from the concentrate but also from the butter added as inhibitor. The amount of vitamin present in summer fats was, of course, much more than in winter fats. The degree of inhibition by summer fats was therefore even greater than would appear at first sight.

Table II.

		Immediate blue reading	Blue reading after 30 secs.
Concentrate with 0.4 cc. of butter fat	1932 May 3-4	6	3
	May 10-16	3.5	3
	June 28	3	3
	July 12-Aug. 2	2.5	2.5
	Sept. 30-Oct. 13	2.5	2.7
	Oct. 31-Nov. 3	3.5	3.0
	1933 Jan. 26-30	5.5	2.5
	Feb. 24-27	6	2
	March 24-27	6	2
	April 19-20	6	2.5
Concentrate alone	April 27-28	4	2.5
	May 9-11	3	2.5
		7	6.5

Fig. 2. Seasonal variation in the inhibitory power of butter-fat on the SbCl_3 reaction of added vitamin A concentrate. The continuous curve gives readings immediately after adding reagent, the broken curve gives readings after 30 secs. The concentrate by itself gave a reading represented by the horizontal axis; after 30 secs. the colour faded to correspond with the dotted line just above the horizontal axis. The scale of Lovibond blue readings has been inverted so that the curve rises as the inhibitory power of the butter-fat increases.

DISCUSSION.

The above data on the yellow value of untreated butter-fat and on the blue value of the non-saponifiable residue provide additional confirmation of the already well established seasonal variation in the carotene and vitamin A contents of butter-fat depending on the change in the nutrition of the cow on turning out to grass.

On the other hand the blue value of the untreated butter-fat is almost valueless as a guide to vitamin A content. In this case the intensity of blue colour given by the butter-fat is affected in a positive sense by the amount of vitamin A present, but also in a negative sense by the inhibitory power of the fat. The effect of pasture feeding is to increase both these variables, with the result that the effects cancel out and the observed blue value is little changed¹.

¹ The blue value of the untreated butter-fat may sometimes show a definite increase under abnormal conditions of feeding. Thus Moore [1932] found the blue value to be increased on supplementing the stall dietary with large amounts of carrots. A similar result may be obtained by feeding cod-liver oil.

Apart from the question of following the seasonal variation, the blue value determined on the untreated fat in any case represents only 1/10–1/5 of the true blue value as determined on the non-saponifiable residue.

In regard to the practical problem of assaying the total vitamin A activity of butter-fats by colorimetric methods it is obvious that both carotene and preformed vitamin A must be taken into account. The carotene content, on the assumption that yellow colour is entirely due to this pigment, may be calculated on the basis that pure carotene has a yellow value of about 2000 Y.U. per mg. The vitamin A content, to avoid unnecessary assumptions as to the blue value of pure vitamin A, may be expressed in blue units determined on the non-saponifiable residue¹. On attempting to combine the carotene and vitamin A contents to arrive at the total activity of the butter-fat, however, we are faced with the difficulty that no general agreement has yet been reached on the relative activities of carotene and vitamin A. To help in the interpretation of the present results, and without implying that an accurate ratio has yet been determined, it will be assumed from the recent work of Moore [1933] that 2 Y.U. of carotene are roughly equivalent to 5 B.U. of vitamin A. Acting on this assumption, and for purposes of simplicity accepting the international standard of vitamin A activity as referring to 1γ of pure carotene², the total activity of a given sample of butter-fat might be calculated as follows.

$$\frac{\text{Y.U. per g.}}{2} + \frac{\text{B.U. (N.S.R.)}}{5} = \text{international units per g.}$$

When we apply this formula to typical samples of "winter" (April 19–20, 1933, giving 3.2 Y.U., 36 B.U. per g.) and "summer" (May 19–22, 1933, giving 18 Y.U., 72 B.U. per g.) butter-fats it will be seen that the total vitamin A value is increased from about 8.8 to about 23.4 units per g. The contribution of carotene to the total activity is of course much more substantial in the "summer" sample, representing about 1/3 of the total activity. Biological experiments to decide the actual distribution of activity between carotene and vitamin A in summer butters by means of tests on untreated butter-fat and the same sample after decoloration by treatment with charcoal are now being carried out by two of us (R. G. B. and S. K. K.).

In a subsequent communication we hope to deal with spectroscopic variations in winter and summer butter-fats.

SUMMARY.

1. The seasonal variation in the carotene and vitamin A contents of typical English butter from Shorthorn cows was followed by colorimetric methods. Determinations of the intensity of yellow colour were carried out on the untreated butter-fats. SbCl_3 blue values were determined both on the untreated fats and the corresponding non-saponifiable residues.

¹ Theoretically a correction equal to about 1/10 of the yellow value should be deducted from the blue value of the non-saponifiable residue to account for the amount of blue colour contributed by the carotene present. This correction, however, would appear to be so small as to represent an unnecessary refinement at the present stage of accuracy. In agreement with this view, in the case of most butters very little difference could usually be detected in the blue value of the non-saponifiable residue when determined on the natural yellow fat and on the same sample saponified after the removal of the carotene by absorption on charcoal.

² The international standard carotene is admittedly not of the highest attainable purity, but here again it is obviously premature to suggest a correction until general agreement has been reached.

2. Data obtained on yellow colour and blue value as determined on the non-saponifiable residue were in good agreement with the well established variation of the carotene and vitamin A contents of the butter corresponding with the quantity of grass or of green fodder available in the diet of the cow.

3. Blue values determined on the untreated butter-fat were found to be valueless as a guide to vitamin A content, being 5–10 times lower than values determined on the non-saponifiable matter.

4. Although blue values determined on the untreated fats showed no consistent quantitative variation, qualitative differences in the behaviour of the blue colours produced by "winter" and "summer" butter-fats were observed. These differences were connected with an inhibitory substance or substances, which was present in much larger concentration in "summer" than in "winter" butter-fats, and whose presence could be detected from its inhibitory power on the blue colour given by vitamin A when added from an external source.

5. In the practical assay of the total vitamin A activity of butter-fats by colorimetric means it is necessary that both the vitamin A and carotene contents should be taken into account. A provisional formula for calculating the total vitamin A activity is suggested. The total vitamin A activity of summer butter-fat from Shorthorn cows appears to be some three times greater than that of winter butter-fat. The fraction of the total activity due to carotene is also greater in summer butter-fat.

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