XVII. THE VITAMIN D ACTIVITY OF BUTTER.

II. THE COMPLEX NATURE OF THE ANTIRACHITIC ACTION OF BUTTER.

BY STANISŁAW KAZIMIERZ KON
AND ROLAND GORDON BOOTH.

*From the National Institute for Research in Dairying,
University of Reading.*

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IN the first paper of this series [Kon and Booth, 1933, 2] evidence was brought forward to show that the antirachitic activity of autumn and winter butter cannot be quantitatively recovered in the non-saponifiable residue of these butters. Under exactly similar experimental conditions irradiated ergosterol or cod-liver oil could be subjected to saponification either alone or mixed with winter butter without loss of potency, *i.e.* their non-saponifiable residues carried the whole of their antirachitic activities.

Since then we have studied the effect of saponification on butters produced under summer conditions and also on butters rendered highly antirachitic either by direct irradiation with the quartz mercury vapour lamp, or by the feeding to cows of large amounts of irradiated yeast or of cod-liver oil. We have also attempted to concentrate the antirachitic activity of these various butters and of cod-liver oil by the method of alcoholic extraction used by Zucker [1922-23; 1925; 1928] for the preparation of an active concentrate from cod-liver oil.

EXPERIMENTAL.

Methods.

Most of the methods used for the saponification of butter for the preparation of olive oil dilutions of the non-saponifiable residues and for the animal tests have been described in the previous paper [Kon and Booth, 1933, 2]. The short saponification method was used throughout. For feeding, the non-saponifiable residues, Zucker extracts and other substances of which the doses given were too small for administration by 1 ml. calibrated volumetric pipettes were dissolved in ether and mixed with a small quantity of olive oil. After evaporation of the ether *in vacuo*, the remainder of the requisite amount of olive oil was added, the solution was mixed by the aid of a vigorous stream of nitrogen and fed by means of standardised pipettes delivering drops weighing from 19 to 21 mg. The dilution was so adjusted that the requisite dose of non-saponifiable residue or other material was present in from 1 to 4 drops of the solution.

In the distribution of animals for the feeding tests an endeavour was made to divide the litters as evenly as possible among the various substances undergoing test, and as a rule litter-mates were placed in all groups where comparison was especially desirable, such as the comparison between one level of raw butter and its equivalent as non-saponifiable residue. In these, as well as in the previous experiments [Kon and Booth, 1933, 2], not only was the degree of protection

afforded to rats by the various substances measured by the amount of ash in the dry defatted femora and humeri, but photographs were also taken, by the technique described in our previous paper, of sections of radii and ulnae stained with silver nitrate. The large number of rats used in the present tests precludes the publication of these photographs. The results arrived at by a study of these photographs were, in general, in excellent agreement with those based on the ash percentage.

Experiments carried out in the summer of 1933.

Methods. (a) *Summer butter from cows at pasture.* The cows went out to grass on April 20th, 1933. Butters from seven bi-weekly churnings were collected between May 12th and June 5th and aliquot parts of these butters, after rendering and filtering, were pooled. This period was very sunny throughout, there being an average of 5.6 hours of sunshine daily, to all of which the cows were exposed. The butter was saponified in the usual way on 3 occasions during the course of the experiment, the non-saponifiable residue being extracted with 4 portions of ether instead of the usual 3.

(b) *Butters from cows receiving irradiated yeast*¹. Two groups of 2 Shorthorn cows each were maintained throughout the winter stall-feeding period 1933 on a ration identical with that fed to the typical herd of Shorthorns [Golding *et al.*, 1932]. Towards the end of this period the ration of one group was supplemented with 50 g. of irradiated yeast daily, while the supplement to the other group was 500 g. daily. In the case of group I, after 14 days of such supplementary feeding butter was made from the cream of the mixed milk of the 2 cows over a 2-day period. The butter was made in the case of group II from a similar sample of cream from the mixed milk of the 2 cows after the yeast had been fed for a period of 3 weeks. These butters were rendered and filtered in the usual way and kept in cold storage until required. They are hereinafter referred to as low-level and high-level yeast butter respectively.

(c) *Butter from cows receiving cod-liver oil*¹. The butter was produced at the same time and under the same conditions as that of the cows receiving irradiated yeast. This group of 2 cows was given, however, daily supplements of 4 to 6 oz. of cod-liver oil for one week and 8 oz. daily per cow for 2 following weeks before the butter was collected.

(d) *Irradiated butter.* In preparing this butter we endeavoured to duplicate as far as possible the method of Steenbock and Wirick [1930] with the exception that we used for irradiation a sample of winter butter instead of June butter as used by these authors. The sample of winter butter collected between March 31st and April 4th, 1933, was rendered and filtered in the usual way and stored until required. After melting, 45.36 ml. of the fat were run into a circular bright nickel-plated tray 19 cm. in diameter, thus giving a layer of fat 0.16 cm. thick. The butter was maintained in the fluid state and at a temperature not exceeding 40° by being placed over a hot water-bath during irradiation. This was carried out for $\frac{1}{2}$ hour with a K.B.B. atmospheric type quartz mercury vapour lamp situated at a distance of 45.7 cm. from the melted fat. The lamp had previously been in use for about 200 hours and consumed 2.5 amp. with a potential across the burner of 132 V. After irradiation the butter was kept in cold storage and, when necessary, saponified in the usual way.

(e) *Alcoholic extract of winter butter prepared by a method similar to that of Zucker* [1922-23; 1925; 1928]. 250 g. of pooled butter from bi-weekly samples

¹ The experiments dealing with the feeding to cows of irradiated yeast and of cod-liver oil will be reported in detail elsewhere.

collected between March 13th and 27th, 1933, were heated with 150 ml. of absolute ethyl alcohol until completely dissolved. The solution was vigorously shaken and allowed to cool and separate, and the alcoholic layer was then poured off. This extraction was followed by another with 100 ml. of 96 % alcohol and by two further extractions each with 75 ml. of 96 % alcohol. The combined alcoholic extracts were evaporated on the water-bath yielding 17.7 g. of a yellow oil. A part of this oil was saponified in the usual way. The residue from the alcoholic extraction was heated to about 45° and washed in a separating funnel with three successive portions of 100 ml. of hot water. It was then filtered and stored.

In Table I are given the results of the tests.

Table I. *Summer 1933. Prophylactic experiment. Antirachitic effect of various butters, of non-saponifiable residues from these butters and of an alcoholic extract and residue from one of them. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentage are averages of duplicate determinations on groups of 4 rats.

In the Tables the dosages of non-saponifiable residues, Zucker extracts, etc., are expressed in the equivalent of the butter from which they were derived.

Positive controls			52.0 % ash			Dosage g.	Ash %
Negative controls (7 rats)			31.2 % ash				
Substance tested							
Summer butter						0.1	36.1
"						0.2	42.8
"						0.5	49.5
"						1.0	53.5
Non-saponifiable residue of summer butter, equiv. ...						0.1	36.1
"						0.2	38.7
"						0.5	44.0
"						1.0	49.8
High-level yeast butter						0.01	46.0
"						0.03	50.4
"						0.10	51.1
"						0.25	52.9
Non-saponifiable residue of high-level yeast butter, equiv. ...						0.01	45.2
"						0.03	50.3
"						0.10	50.8
"						0.25	52.4
Low-level yeast butter						0.05	38.0
"						0.15	49.7
"						0.50	53.5
Non-saponifiable residue of low-level yeast butter, equiv. ...						0.05	39.1
"						0.15	46.0
"						0.50	50.6
Cod-liver oil butter						0.10	38.1
"						0.30	48.6
"						1.00	51.8
Non-saponifiable residue of cod-liver oil butter, equiv. ...						0.10	38.0
"						0.30	42.5
"						1.00	48.3
Irradiated butter						0.018	49.8
"						0.036-0.072	50.4
Non-saponifiable residue of irradiated butter, equiv. ...						0.018	49.9
"						0.036-0.072	50.4
Winter butter						1.00	48.0
Zucker extract of winter butter, equiv. ...						1.00	35.2
Non-saponifiable residue of Zucker extract of winter butter, equiv.						1.00	35.1
Residue of winter butter after Zucker extraction, equiv. ...						1.00	46.9

It is apparent from the table that neither the high-level yeast butter nor the irradiated butter has lost its potency as a result of saponification. In the case of the irradiated butter the experiment is unfortunately rather unsatisfactory. Owing to a misjudgment of the degree of protection afforded to the rats receiving the irradiated butter it was decided on the 15th day of the experiment to double the dosage in the case of the animals receiving the higher level of butter and obviously also in the case of the animals receiving the corresponding level of non-saponifiable residue. When the rats were killed it was found that the earlier diagnosis *in vivo* was quite erroneous and that not only the increased higher dosage but also the lower dosage had afforded a high degree of protection. It is therefore possible that, in the case of this butter, some destruction of the antirachitic factor might have taken place upon saponification, but that since the lower dosage was almost sufficient for complete protection, any slight destruction of antirachitic factor would not be apparent.

In the case of the low-level yeast butter, no destruction is evident at the lowest dosage. On the other hand both the 0.15 and 0.5 g. levels of this yeast butter yield a definitely higher ash percentage than their corresponding non-saponifiable residues.

A very similar condition prevails in the case of the cod-liver oil butter. There is no difference between butter and non-saponifiable residue at the 0.05 g. level, but here again, two higher dosages of butter yield higher ash percentages than their corresponding non-saponifiable residues.

The same statement holds true in the case of summer butter.

It will be noticed that the antirachitic activity of summer butter withstands saponification to a greater extent than that of autumn and winter butters. Our observations on butters collected in summer 1933 are in complete agreement with a preliminary test carried out on butter collected in the summer period 1932 previously recorded by us [Kon and Booth, 1933, 1].

With regard to the Zucker extract of winter butter it is quite evident that, as far as this experiment goes, it would confirm Zucker and Barnett's opinion [1922-23], quoted in our previous paper [Kon and Booth, 1933, 2], that the antirachitic activity of butter cannot be concentrated by their method. It is obvious that the loss is not due primarily to destruction of the antirachitic factor by saponification, as practically the whole of the activity was left in the residue, and the saponification did not in this case further diminish the already slight activity of the alcoholic extract.

Experiments carried out in autumn 1933, first series.

In this period we repeated our summer experiments with irradiated butter using dosages of 5 and 10 mg. These dosages were intended to afford an incomplete protection in order to obtain an unequivocal result as to whether or not destruction occurred in this butter on saponification. We also prepared and tested in this period Zucker extracts of high-level yeast butter, irradiated butter, winter butter and cod-liver oil. It was obvious that before reaching any definite conclusions with regard to the inapplicability of Zucker's method to butter, we had to make sure that we could obtain by this method, and under our laboratory conditions, an active concentrate from cod-liver oil. As an additional check on our previous findings as to the effect of saponification upon the antirachitic potency of various butters we again saponified a sample of winter butter.

Methods. (a) *Irradiated butter.* The same sample was used as in the previous summer experiment. This had been kept in the ice-chest throughout the intervening period. The methods of saponification and of feeding the butter and its

non-saponifiable residue were exactly as previously described, but four ethereal extractions of the non-saponifiable residue were made from the saponification mixture.

(b) *Preparation of the Zucker extracts.* For the preparation of Zucker extracts we used the same high-level yeast butter and irradiated butter as in our summer experiment. The cod-liver oil was from the bulk given us by Dr Coward for use in our previously reported experiments [Kon and Booth, 1933, 2], while the winter butter was from a pooled sample collected bi-weekly between February 10th and March 14th, 1933. The Zucker extracts of all these substances were made in exactly the same way. 20 g. of the substance to be extracted (200 g. in the case of winter butter) were mixed with 25 ml. of absolute alcohol (250 ml. in the case of winter butter) and were shaken on a mechanical shaker for $1\frac{1}{2}$ hours. The butter-alcohol mixtures were, before shaking, heated sufficiently to melt the butter-fat. In all, four extractions were made, the same amount of alcohol being used for the second extraction as for the first, while in the last two extractions the amount of alcohol used was halved. After each of the first three extractions the oils were allowed to settle, and the alcoholic layer was siphoned off; after the last extraction the mixtures were centrifuged before the alcoholic layer was siphoned off. In each case the bulked extracts were concentrated on the water-bath, while the residues were washed four times with hot distilled water. The following yields were obtained:

Cod-liver oil	2 g. extract from 20 g. original oil.
Irradiated butter	5.08 g. extract from 20 g. original butter-fat.
High-level yeast butter	5.69 g. extract from 20 g. original butter-fat.
Winter butter	52.15 g. extract from 200 g. original butter-fat.

No special effort was made to recover the residues quite quantitatively, as the amounts used were far in excess of the requirements for the feeding tests, but for the purposes of dilution and feeding it was always assumed that the weight of the residue was equal to the weight of the original substance less the weight of the extract. It will be noticed that this method of extraction, which follows almost exactly that recommended by Zucker [1925; 1928], gives a much greater yield of extract from butter than the modification used in our summer experiment.

The methods of saponification and feeding of the Zucker extracts and residues and of their non-saponifiable residues were as previously described. The same applies to the saponification and feeding of winter butter; in all cases the soaps after saponification were extracted with four successive quantities of ether.

In Table II are given the results of these tests.

It can be seen that neither of the two levels of irradiated butter fed this time was sufficient to bring about complete protection. While the non-saponifiable residue of the lower level gives a slightly lower percentage of ash than the butter itself the reverse is true in the case of the higher level, and as these differences are well within the experimental error it is obvious that the antirachitic factor of irradiated butter can be quantitatively recovered in the non-saponifiable residue of the latter.

The saponification of winter butter has again resulted in a very incomplete recovery of its antirachitic activity in the non-saponifiable residue.

The results with the Zucker extracts have been rather unexpected. It is obvious from our figures that our inability to extract the vitamin D potency by this method is not restricted to winter butter alone, or even to butters of an enhanced antirachitic potency completely recoverable after saponification, but extends to cod-liver oil. It will be noticed that, while in the case of the latter the

Table II. *Autumn 1933. Prophylactic experiment, first series. Antirachitic effect of Zucker extracts and residues from cod-liver oil and various butters before and after saponification; antirachitic effect of the original butters and cod-liver oil and of the non-saponifiable residues of two of the butters. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentages are averages of duplicate determinations on groups of 4 rats.

Negative controls 34.9 % ash

Substance tested	Dosage g.	Ash %
High-level yeast butter	0.010	44.8
Zucker residue of high-level yeast butter, equiv.	0.010	33.7
Zucker extract of high-level yeast butter, equiv.	0.010	35.7
Non-saponifiable residue of Zucker extract of high-level yeast butter, equiv.	0.010	35.9
Cod-liver oil	0.0008	48.4
Zucker residue of cod-liver oil, equiv.	0.0008	44.7
Zucker extract of cod-liver oil, equiv.	0.0008	32.9
Non-saponifiable residue of Zucker extract of cod-liver oil, equiv. ...	0.0008	33.9
Irradiated butter	0.005	44.8
"	0.010	46.9
Non-saponifiable residue of irradiated butter, equiv.	0.005	43.7
"	0.010	47.6
Zucker residue of irradiated butter, equiv.	0.010	35.9
Zucker extract of irradiated butter, equiv.	0.010	37.9
Non-saponifiable residue of Zucker extract of irradiated butter, equiv.	0.010	38.0
Winter butter	0.700	47.3
Non-saponifiable residue of winter butter, equiv.	0.700	35.4
Zucker residue of winter butter, equiv.	0.700	37.2
Non-saponifiable residue of Zucker residue of winter butter, equiv. ...	0.700	33.6
Zucker extract of winter butter, equiv.	0.700	36.4
Non-saponifiable residue of Zucker extract of winter butter, equiv. ...	0.700	33.1

residue after extraction seems to have retained a large part of its antirachitic potency, in the case of all the butters both the extract and the residue are only very slightly active. But even here the extract and residue of winter butter have suffered further loss on saponification.

Shipley *et al.* [1924] report that alcoholic extracts of butter-fat may contain appreciable amounts of antirachitic potency while the alcohol-insoluble residue was found by them to be inactive.

Experiments carried out in autumn 1933, second series.

Autumn butter collected in this period was tested for its antirachitic potency before and after saponification. The results of these tests were again in complete agreement with our previous experience with autumn and winter butters. At the same time, other experiments were carried out. These will be reported in the following paper.

Methods. The butter used in this experiment was collected between October 16th and November 16th, 1933. Five saponifications were carried out, the last four on the previous week's churnings, while in the first one a 3 days' sample was used. In order to ensure as perfect a recovery as possible the non-saponifiable residue was extracted on these occasions 5 times with ether.

The autumnal rains which followed the very dry and hot summer of 1933 had produced an exceptionally good crop of grass for this time of the year, and the butters, especially the first 2 or 3 samples, were therefore much more highly coloured than is usual under our conditions at this season. In fact the yellow

pigmentation and the vitamin A content of these butters were higher than those of the summer butter tested in the first series of experiments reported in this paper. On the other hand the actual sunshine to which the cows were exposed was in this case only 3·16 hours daily. The intensity of solar radiation at this time of the year was also obviously less than in summer. Observations on the colour and vitamin A activity of these butters will be reported in detail elsewhere. It might be mentioned here, incidentally, that the depth of yellow coloration and/or of vitamin A activity of a butter cannot be used as a reliable guide to its vitamin D activity.

In Table III are given the results of the tests.

Table III. *Autumn 1933. Prophylactic experiment, second series. Antirachitic effect of autumn butter and of its non-saponifiable residue. Percentage of ash in dry defatted femora and humeri.*

The figures for ash percentages are averages of duplicate determinations on groups of 4 rats.

Positive controls (2 drops of c.l.o. daily)		52·3 % ash
Negative controls		33·2 % ash
Substances tested	Dosage g.	Ash %
Autumn butter	0·25	40·8
"	0·50	47·1
"	1·00	52·9
Non-saponifiable residue of autumn butter, equiv. ...	0·25	34·6
"	0·50	39·1
"	1·00	40·3

This autumn butter is more potent than that collected at the same time during the previous year; on the other hand it is less potent than the sample of butter collected and tested in summer 1933. The loss of potency after saponification is quite marked and, as the non-saponifiable matter had been in this case so thoroughly extracted, this loss of potency in the course of saponification must be real and cannot be due to faulty extraction.

Experiments carried out in autumn 1933, third series.

The experiments carried out in this series were chiefly of a routine nature and formed part of our regular estimation of the antirachitic factor of butter by the curative line test technique. This latter has been described in our previous paper [Kon and Booth, 1933, 2]. The autumn butter was fed at three levels: 0·25, 0·5 and 1·0 g. daily before and after saponification.

The two lower levels of butter failed to give any definite signs of healing while 4 out of 5 rats receiving 1·0 g. of butter showed such signs varying in intensity from an indication of a line to a medium broken line. The lower potency of the non-saponifiable residue is shown by the fact that in only 2 out of 5 rats receiving the highest level are specks of calcification to be found.

DISCUSSION.

Before devoting our attention to what we believe to be the positive results of our work, we would like to discuss briefly a negative finding, *i.e.* our inability to obtain an active extract from cod-liver oil by the method employed by Zucker [1922-23; 1925; 1928]. When in the summer of 1933 we prepared the Zucker extract

from butter and found it inactive we thought that this finding very definitely lent support to our contention that a chemical difference exists between the antirachitic agent of butter and other antirachitic agents. Obviously the validity of this finding stood or fell with our ability to obtain by the same method an active extract from cod-liver oil. Our failure to obtain such an extract is definitely not due to a destruction in the course of extraction, as the residue after extraction was found to contain the major part of the original activity. That highly active concentrates can be, and are, prepared from cod-liver oil by the method of Zucker we do not doubt. Such extracts are produced under Zucker's patents [Zucker, 1925; 1928] on a large scale in the United States and are, as Zucker [1933, 1, 2] reports, regularly used for enhancing the antirachitic potency of bread and milk.

We do not know what grades of cod-liver oil are used in this process. The cod-liver oil unsuccessfully used by us was one of very high vitamin D content (250–300 International units per g.) and was a high grade medicinal oil kindly given us by Dr Coward. It is conceivable that the acidity of the oil may play a vital part in the process of extraction, and that vitamin D could be extracted from oils of high acidity but not from neutral oils. This is merely an assumption, but the fact remains that our results in this respect are of no use in elucidating the difference between the antirachitic factor of butter and that from other sources.

In our first paper [Kon and Booth, 1933, 2] we reported observations on the effect of saponification on butters collected in autumn and winter. We observed that only a small fraction of the original antirachitic activity of these butters could be recovered in the non-saponifiable residues. At that time we abstained from making any suggestions regarding the possible abnormal behaviour of the antirachitic activity of butters in general, especially as we had been led by preliminary experiments [Kon and Booth, 1933, 1] to believe that butters may differ in this respect. Since then we have examined a variety of butters, and we have found that while the activity of some of them is markedly reduced by saponification, the antirachitic potency of others will withstand this treatment just as well as those of cod-liver oil and irradiated ergosterol.

When the butters examined by us are placed in an ascending series according to the degree of recovery in the non-saponifiable residue of their original antirachitic activity, it becomes at once evident that these butters are also graded in another respect—namely that they have been simultaneously arranged in ascending order of original antirachitic potency. In other words, the greater the potency of the butter, the more complete in our experience is the recovery of this potency after saponification.

The loss of potency on saponification is never complete, a loss of about 80 % of the original potency seems to be the maximum we have experienced. Such is the case with the least potent of the butters collected in autumn and winter. Any increase of potency above this practically basal level is accompanied by a corresponding, and, apparently, quantitative increase in the activity recoverable after saponification. We are therefore led to believe that at least two factors antirachitic for the rat are present in the butters which we have so far examined. One of them is clearly destroyed or diverted into a fraction other than the non-saponifiable residue during the course of saponification. The other behaves in an orthodox way. Whether this latter factor is to be identified with the vitamin D of cod-liver oil, whether it is the same as irradiated ergosterol, or whether, possibly, it differs from those two recognised factors, we are at present obviously not in a position to say. We only know that it shares with those two factors their resistance to saponification. It has been so far our experience that, by whichever method we

attempted to increase the antirachitic activity of the butter, the added factor behaved in a normal way.

It would therefore appear that while the cow possesses the ability of secreting into her milk not only vitamin D but also another substance or property antirachitically active for the rat, the yield of this substance cannot be influenced either by changes in the natural conditions such as changes from winter to summer types of feeding, or by massive feeding of highly potent carriers of vitamin D activity, or, lastly by direct irradiation of the butter. The cow thus appears to be able to produce a novel antirachitic agent, but definitely does not rely for its production upon any exogenous source of vitamin D *sensu stricto*, whatever this term implies. A physiological conversion of added known forms of vitamin D into the novel form found in butter has not, in our experience, been found to occur.

As far as we can judge, this factor would seem constant in all the butters and always present in the same amount in a given bulk of butter. Thus, for example, it might constitute 75 % of the antirachitic activity of a butter of which 1 g. daily is required to give almost complete protection. When on the other hand the activity of such a butter is raised nearly a hundred times and 10 mg. of this butter nearly suffice to afford complete protection, the orthodox factor only is increased and the rôle played by the unresistant factor becomes so small that its loss on saponification is not noticeable.

SUMMARY.

1. The following butters have been tested for their antirachitic potency both before and after saponification: (a) butter from cows on summer pasture, (b) butters from cows having received two different levels of irradiated yeast, (c) butter from cows having received cod-liver oil, (d) irradiated winter butter. It was found that the antirachitic value of high-level yeast butter and of irradiated butter could be recovered practically completely in the non-saponifiable residue, while the remaining butters suffered a more or less marked loss in the course of saponification. These butters, however, including summer butter, were definitely more resistant to saponification than the autumn and winter butters reported on in the previous paper of this series.

2. In confirmation of previous work it was found that a large part (over 75 %) of the potency of autumn and winter butters was either destroyed or diverted to another fraction on preparation of the non-saponifiable residues.

3. The greater the initial antirachitic potency of the butter, the greater is the proportion of the potency recoverable in the non-saponifiable residue.

4. It is suggested that at least two factors antirachitic for the rat are present in butter; a factor which is not recoverable in the non-saponifiable residue, and the usual vitamin D (whatever this term may imply), which is resistant to saponification. By exposure to summer conditions or by the feeding to cows of highly antirachitic substances such as irradiated yeast or cod-liver oil the concentration of the stable factor but not of the labile factor of butter can be markedly increased. Direct irradiation of the butter also leads to the formation of the stable factor only.

5. Attempts to achieve a chemical separation of the labile and stable antirachitic factors of butter by submitting butters of varying potency to extraction with alcohol, following the method of Zucker, failed in so far as we were unable, contrary to Zucker's findings, to extract by this method the antirachitic potency of a high grade cod-liver oil, or, in agreement with Zucker, to extract that of butter.

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