CLXXVII. THE VITAMIN D ACTIVITY OF BUTTER.

I. A CHEMICAL DIFFERENTIATION OF THE ANTI- RACHITIC FACTOR OF AUTUMN AND WINTER BUTTER FROM IRRADIATED ERGOSTEROL AND THE VITAMIN D OF COD-LIVER OIL¹.

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IN the course of a study of the vitamin content of typical English milk carried out in the National Institute for Research in Dairying it has been our custom to estimate vitamin D in butter in winter, summer and autumn by curative and protective experiments on rats.

As is well known, the vitamin D content of butter is at its lowest during the winter months. In 1931-32 we found that 1.5 g. of our winter butter fed daily over a period of 10 days to rachitic rats did not suffice to bring about even a partial cure of the rachitic condition, that is, we were unable to obtain even a faint "line" with this amount in the curative line test. In our technique the butter-fat is administered to the experimental animals by pipette separately from the diet, and we find it practically impossible to give to the rats more than 1.5 g. (or almost 2 cc.) of butter daily.

When planning the curative experiments for the next winter period (1932-33) we decided to concentrate the antirachitic factor of butter by saponification and to feed to rats the non-saponifiable residue of larger quantities of butter, 3 and 6 g. daily. In order to test the feasibility of such a method we decided to rehearse it in the preceding autumn and to compare, in protective experiments, the antirachitic effects of graded amounts of autumn butter with those of equivalent amounts of the non-saponifiable residue from this butter. The protective technique was chosen in preference to curative tests because, when equal numbers of experimental animals are used, it is, in our opinion, more accurate than the latter and therefore more likely to show any possible defects in our method.

EXPERIMENTAL.

Experiments carried out in autumn 1932.

Method of saponification of butter. The butter used in this study was obtained from a herd of shorthorns belonging to this Institute. The care and management of this herd are typical of the agricultural practice in the south of England [Golding *et al.*, 1932]. The butter was churned twice weekly in the Institute's dairy from fresh cream separated from mixed samples of evening's and morning's

¹ Read before the Biochemical Society, May 6th, 1933 [Kon and Booth, 1933].

milk blended in the proportion of 2:3, and was unsalted. It was melted at a temperature not exceeding 80° practically immediately after delivery from the dairy, and the rendered fat, after filtration, was stored in the ice-chest until required. The saponification was carried out as follows: to 50 g. of butter-fat 100 cc. of absolute ethyl alcohol were added, followed by 22 cc. of a saturated aqueous solution of KOH (80 g. A.R. dissolved in 54 cc. of water). The mixture was boiled on the water-bath for 1 hour, 400 cc. of water were added and the non-saponifiable matter was extracted by shaking with three 250 cc. portions of "anaesthetic" ether. The ethereal extract was washed twice with 200 cc. of water, dried over anhydrous Na_2SO_4 , the bulk of the ether driven off on the water-bath and the residue taken to dryness *in vacuo*. The non-saponifiable residue, usually amounting to about 200–250 mg., was, after weighing, dissolved in olive oil to a total weight of 5 g. This was done by dissolving the non-saponifiable residue in a small amount of ether, adding the requisite amount of olive oil and driving off the solvent *in vacuo*. In this way 1 g. of the olive oil solution contained the non-saponifiable residue from 10 g. of butter. For feeding, the oily solution was distributed, by means of calibrated pipettes, into small porcelain dishes. The butter was saponified twice weekly, 10 samples churned between October 25th and December 3rd, 1932, being used altogether for the autumn experiment. Of these the first six were distributed for feeding as described above. In the case of the last four the procedure was slightly modified, in that, after the non-saponifiable residue had been incorporated in olive oil, the solution instead of being distributed as such, was dissolved in 50 cc. of A.R. benzene. The benzene solution was then distributed into porcelain dishes from a burette. The solvent was evaporated before a fan and the last portions removed in a vacuum desiccator. The raw butter used for feeding was always from the same sample as that used for saponification.

Animal tests. For the feeding test young rats weighing 55–65 g. were used. They were left with their mothers until they reached that weight (generally between the 21st and 25th day of life). They were then randomised, placed on the 2965 rachitogenic diet of Steenbock, and the various substances to be tested for their antirachitic potency were given from the start separately from the diet for a period of 5 weeks, six times a week. At the end of the experimental period the rats were killed by coal gas, and the antirachitic potency of the substances tested was assessed by estimating the ash content of dry defatted femora and humeri and also by the appearance of sections of radii and ulnae stained with silver nitrate. Four rats were used for every substance or level tested.

In Table I we give the ash percentages obtained for several levels of autumn butter and for equivalent amounts of the non-saponifiable residue from this butter, while Plate III, fig. 1, gives photographs of stained sections of radii and ulnae of rats having received the highest level of butter and non-saponifiable residue, taken by the method of Dyer [1931] slightly modified¹.

It is obvious both from the table giving the percentages of ash and from the appearance of sections of radii and ulnae that, as a result of saponification, the antirachitic potency of butter had dropped very considerably, the non-saponifiable residue of 1½ g. of butter producing an effect only slightly superior to that given by 0.2 g. of raw butter.

It appears therefore that while these results were quite unsatisfactory so far as the elaboration of a method for the testing of butters of low antirachitic

¹ The bones, mounted according to Dyer's method, were photographed under ½ watt electric illumination, a Wratten K. 3 filter being used and the Ilford panchromatic plates being developed for only 3½–4 mins. in metol-quinol.

Table I. *Autumn 1932. Prophylactic experiment. Antirachitic effect of butter and of non-saponifiable residue from butter. Percentage of ash in dry defatted femora and humeri.*

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

Negative controls 29.2 %.			
0.1 g. butter	31.0 %		
0.2 g. "	33.6	N.S.R. from 0.2 g. butter	31.4 %
0.5 g. "	40.2	" 0.5 g. "	31.4
1.0 g. "	50.0	" 1.0 g. "	33.7
1.5 g. "	51.8	" 1.5 g. "	34.5
Positive controls 52.4 %.			

potency is concerned, they were highly valuable in yielding the unexpected observation that the antirachitic factor of butter does not share with other antirachitic substances their well-known resistance to saponification.

Experiments carried out in winter 1933.

In the following winter period three series of experiments were carried out. In the first series the antirachitic potency of winter butter was compared at several levels in protective experiments with that of the non-saponifiable residue of the same butter, prepared by two different methods. A mixture of irradiated ergosterol (the International Standard of vitamin D) and of the same butter was also subjected to saponification and the antirachitic potency after saponification compared with that of an equivalent dosage of the International Standard of vitamin D (0.4 unit daily).

In the second series two samples of cod-liver oil and the International Standard of vitamin D were saponified both alone and mixed with butter and the antirachitic potency of the non-saponifiable residues was compared in curative tests with that of equivalent amounts of the original substances.

In the third series, the effect of saponification on the antirachitic potency of cod-liver oil and of the International Standard of vitamin D (either alone or mixed with butter) was judged in protective experiments.

First series of winter experiments. As already mentioned butter for these experiments was saponified by two methods, one being the method previously described. In the other, instead of boiling the butter for 1 hour with alcoholic potash we brought the mixture to a temperature of 60–70° for 2 minutes only, saponification being completed under these conditions. As previously, the butter was saponified twice weekly in 50 g. lots, or, in the case of butter saponified for 1 hour in 25 g. lots. The further treatment was as previously described. For feeding, the non-saponifiable residue was dissolved in "anaesthetic" ether and transferred to a volumetric flask containing olive oil to the extent of 10 % by weight of the butter originally taken for saponification. The ethereal solution was made up to contain the equivalent of 1 g. of butter in 1 cc. It was then distributed for feeding into small porcelain dishes from a special burette for volatile liquids. Ether was used in preference to benzene because of the relatively greater ease with which the former solvent may be removed. The butter for this experiment was churned between February 10th and March 14th, 1933.

For saponification the International Standard of vitamin D (VD. 5 issued September 30th, 1932) was mixed with winter butter in the proportion of 1 unit of the Standard to 0.25 g. of the mixture, *e.g.* 76 mg. of the Standard were mixed with 18.924 g. of butter. The mixture was then saponified for 1 hour and the

non-saponifiable residue extracted by a method exactly similar to that used for the extraction of the non-saponifiable residue in the first experiment mentioned. For feeding, the non-saponifiable residue was dissolved in ether and distributed exactly as described in the case of winter butter.

The ash percentages obtained in this experiment are given in Table II, while in Plate III, figs. 2*a* and 2*b* are given photographs of stained sections of radii and ulnae of the experimental animals.

Table II. *Winter 1933. First prophylactic experiment. Antirachitic effect of butter, of the non-saponifiable residue from butter and of the International Standard of vitamin D before and after saponification (mixed with butter). Percentage of ash in dry defatted femora and humeri.*

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

Negative controls 32.2 %.			
0.25 g. butter	37.1 %	N.S.R. from 0.25 g. butter	35.3 %
0.5 g. "	46.0	" 0.5 g. "	36.3
1.0 g. "	50.7	" 1.0 g. "	39.7
1.5 g. "	53.2	" 1.5 g. "	39.2
N.S.R. from 1.5 g. butter saponified for 1 hour			41.2 %
0.2 unit of International vitamin D standard			46.9
0.4 unit of International vitamin D standard			50.2
0.4 unit of International vitamin D standard			
mixed with butter and saponified ...			50.0
Positive controls 50.7 %.			

It will be seen that, as far as butter is concerned, the results are very similar to those obtained in the preceding autumn.

Here again the antirachitic effect of the non-saponifiable residue from 1.5 g. of butter is only slightly superior to that given by the lowest level of raw butter, and the milder saponification does not prevent the loss of potency to any extent.

On the other hand the antirachitic activity of the International Standard of vitamin D has survived unimpaired a saponification in the presence of butter.

Second series of winter experiments. As this series overlapped the first series, the same butter, saponified butter and saponified mixture of butter and International Standard of vitamin D were used in the earlier part of this experiment (butters churned between March 10th and March 14th, 1933) as in the first series. Following on this, butters churned between March 14th and 21st were tested. In addition, two samples of cod-liver oil were used, one kindly given us by Dr K. H. Coward and assessed by her as containing 250–300 International units of vitamin D per g. For the other, one of us (S. K. K.) is indebted to Dr A. D. Holmes of the E.L. Patch Co. in Boston who sent him several samples of this oil in 1929. At that time its potency was given by Dr Holmes as in excess of 125 units (as defined in the E.L. Patch Laboratories and similar to the Steenbock unit) per g.

The cod-liver oils were tested, either as received, or after saponification, either alone or mixed with butter. The raw cod-liver oils were diluted with olive oil so as to contain the requisite amount in 20 mm.³ and were fed from pipettes made in accordance with the specifications of Memorandum 10 (A) of the Dept. of Biological Standards of the National Institute for Medical Research. Dr Coward's cod-liver oil (No. 1) was fed at a level of 2 mg. daily, while of Dr Holmes's oil (No. 2) 8 mg. were given daily. The cod-liver oils were saponified for 1 hour under precisely the same conditions as previously employed for the

butter. Two saponifications of each of the cod-liver oils were carried out in the course of the 10-day feeding period. In view of the much higher activity of cod-liver oil the amounts of non-saponifiable residue fed were much smaller than in the case of butter, and for this reason the concentrates from cod-liver oil were mixed with a relatively larger volume of olive oil and likewise with a relatively larger amount of ether. Otherwise they were distributed in porcelain dishes in exactly the same way as in the case of butter.

As already mentioned, the saponified mixture of butter and irradiated ergosterol was obtained from samples used in the first series of winter experiments.

The International Standard of vitamin D alone was saponified for 1 hour under conditions similar to those described above, the only difference being that, owing to the small quantity of the substance used (1 g.), relatively more ether was used for the extraction of the non-saponifiable residue from the soap solution in order to avoid the increased losses which might occur when such small quantities were handled. Only one saponification was carried out and the olive oil solution of the non-saponifiable residue was stored in the porcelain feeding dishes in the ice-chest.

All these substances were tested on rats in curative tests, *i.e.* they were fed during the course of 10 days to rats previously rendered rachitic by being kept for 21 to 23 days on the 2965 diet of Steenbock. In Plate III, figs. 3 *a*, 3 *b* and 3 *c*, are given photographs of line tests carried out on these rats.

It will be seen that, while the two samples of cod-liver oil and the International Standard of vitamin D have retained their antirachitic potency after saponification, whether saponified alone or in the presence of butter, winter butter has again lost at least a large part of its original potency. Incidentally the butter was more potent this year than in the preceding winter and gave a definite line at the 1.5 g. level.

Third series of winter experiments. In the third series of experiments the International Standard of vitamin D (V.D. 6, distributed March 31st, 1933) was fed at 3 levels of 0.1, 0.15 and 0.2 unit daily and its antirachitic potency compared in protective experiments with that of the saponified Standard fed at the 0.2 unit level and with that of a saponified mixture of the Standard with winter butter also fed at the 0.2 unit level.

The Standard was saponified as previously described. The mixture of butter and Standard was made in the proportion of one part of the International Standard to 100 parts of the mixture; it was saponified in the usual way. At first the non-saponifiable residues were dissolved in ether and distributed as previously described. Later (10 days after the commencement of the experiment) the method was modified in the following way. The non-saponifiable residues were dissolved in a small quantity of ether and mixed with olive oil in such a way that the requisite amount of the residue was present in 20 mm.³ of the solution after evaporation of the ether. These olive oil solutions were then fed by means of vitamin D pipettes. The bulk was kept in the ice-chest, and the methods suggested by the Department of Biological Standards of the National Institute for Medical Research for the handling of the International Standard of vitamin D and of its dilutions were rigidly adhered to.

Dr Coward's cod-liver oil was fed as such after dilution with olive oil (0.8 mg. of cod-liver oil daily) and was also fed after being saponified either alone or mixed with butter in the proportion of 2.5 g. of oil to 97.5 g. of butter. These non-saponifiable residues were fed in exactly the same way as those of the International Standard of vitamin D.

1.5 g. of autumn butter. Prophylactic experiment.

Non-saponifiable residue from 1.5 g. of autumn butter.

Fig. 1.

1.5 g. of winter butter. Prophylactic experiment.

Non-saponifiable residue from 1.5 g. of winter butter.

Fig. 2a.

0.2 unit of the International Standard of vitamin D.
Prophylactic experiment.

0.4 unit of the International Standard of vitamin D.

Fig. 2b.

1.5 g. of winter butter. Curative experiment.

Non-saponifiable residue from 1.5 g. of winter butter.

Fig. 3a.

8 mg. of cod-liver
oil No. 2.

2 mg. of cod-liver oil No. 1.

Non-saponifiable residue
of 8 mg. of cod-liver oil
No. 2.Non-saponifiable residue of 2 mg.
of cod-liver oil No. 1. Non-saponifiable residue
of mixture of 8 mg. of cod-
liver oil No. 2 and butter.Non-saponifiable residue of mixture
of 2 mg. of cod-liver oil No 1 and
butter.

Curative experiments.

Fig. 3b.

0.5 unit of the International Standard of vitamin D.
Curative experiment. Non-saponifiable residue of 0.5 unit of the International
Standard of vitamin D.

Fig. 3c.

Negative Controls. Prophylactic experiment.

0.1 unit of the International Standard of vitamin D.

0.15 unit of the International Standard of vitamin D.

0.2 unit of the International Standard of vitamin D.

Non-saponifiable residue of 0.2 unit of the International
Standard of vitamin D. Non-saponifiable residue of mixture of 0.2 unit of International
Standard of vitamin D and butter.

Fig. 4a.

0.8 mg. of cod-liver oil No. 1. Prophylactic experiment.

Non-saponifiable residue of 0.8 mg. of cod-liver oil No. 1.

Non-saponifiable residue of mixture of 0.8 mg. of cod-liver oil
No. 1 and butter.

Fig. 4b.

For testing these substances 3 rats were used for each level (instead of the usual 4) and only 2 were available for the 0.1 unit level of the International Standard.

The ash percentages are given in Table III while photographs of stained sections of the wrist bones will be found on Plate III, figs. 4 *a* and 4 *b*. It is

Table III. *Winter 1933. Second prophylactic experiment. Antirachitic effect of cod-liver oil and of the International Standard of vitamin D before or after saponification (alone or mixed with butter). Percentage of ash in dry defatted femora and humeri.*

Ash % given as averages of duplicate determinations on groups of 3 rats (2 in the case of the 0.1 unit level of the International Standard).

Negative controls 32.7 %.					
0.1 unit of International vitamin D standard	...	...	...	...	44.9 %
0.15 unit of International vitamin D standard	...	...	...	...	48.8
0.2 unit of International vitamin D standard	...	...	...	...	47.9
0.2 unit of International vitamin D standard after saponification	...	...	...	...	45.5
0.2 unit of International vitamin D standard mixed with butter and saponified	...	...	...	...	46.0
0.8 mg. cod-liver oil	...	...	...	...	48.7
0.8 mg. cod-liver oil after saponification	...	...	...	...	49.4
0.8 mg. cod-liver oil mixed with butter and saponified	...	...	...	...	49.7

evident that, under our experimental conditions, the antirachitic principle (or principles) of cod-liver oil and of the International Standard of vitamin D is not affected by saponification in the presence or absence of butter.

DISCUSSION.

When the results of several independent series of experiments described in the experimental part of the present paper are compared and reviewed as a whole, the following general conclusion may, in our opinion, be safely drawn, namely, that the antirachitic agent, whatever its nature, present in winter and autumn butters can, in experiments on rats, be chemically differentiated from irradiated ergosterol as present in the International Standard of vitamin D and from vitamin D *sensu stricto* as it is present in cod-liver oil. Under our experimental conditions we are unable to concentrate in the non-saponifiable residue the antirachitic potency of butter, whereas, under exactly similar conditions, we are able to concentrate in the same fraction practically the whole of the antirachitic activity of the two other antirachitic agents, thus simply confirming in the latter case, for our experimental conditions, the experience of countless other workers. Whether we are dealing here with a destruction of the antirachitic factor of butter or whether it only finds its way into a different fraction at some stage during the course of the preparation of the non-saponifiable residue we are at present unable to say. Experiments on this point are now in progress. Whatever the nature of the change it seems to be brought about readily in the course of saponification as evidenced by our failure to obtain any better concentration of the antirachitic factor in the non-saponifiable residue by using a milder method of saponification.

From our experiments, in which cod-liver oil and irradiated ergosterol were saponified in the presence of butter, we conclude that butter does not contain a specific destructive substance alongside the classic vitamin D, as the presence of butter does not affect the stability to saponification of other antirachitic agents. On the other hand it might be conceived that a specific adjuvant is

present in butter and enhances greatly the pharmacological action of vitamin D *sensu stricto*. While such a possibility seems to us very remote we are not prepared to rule it out without experimental evidence. This we are seeking at present in experiments designed for the purpose.

The discovery of the antirachitic activation of foodstuffs by ultra-violet irradiation and the subsequent work showing that ergosterol was apparently specific in its capacity for activation by this means have led many students of nutrition to believe that only one antirachitic substance exists and to reduce, therefore, all manifestations of antirachitic activity to the common denominator of irradiated ergosterol. Recent evidence has extensively undermined this view, in fact has rendered it untenable. The work of Hess [Hess and Supplee, 1930; Hess, Weinstock and Rivkin, 1930] and of Steenbock *et al.* [1932], to mention only two of the numerous investigators in this field, has shown beyond reasonable doubt that the biological activity of fish-liver oils is, for human beings and birds (chicken), rat-unit for rat-unit, much higher than that of irradiated ergosterol.

Our experiments on butter point to a chemical difference between winter and autumn butter on the one hand and irradiated ergosterol and cod-liver oil on the other as far as their vitamin D activities are concerned. If we accept a difference as existing between the two latter factors, and this we have no reason to deny, it seems to us that the existence of at least a trinity of antirachitic agents is now supported by a respectable weight of evidence.

After having failed to concentrate the vitamin D activity of butter in its non-saponifiable fraction we searched the literature in order to see whether findings of a similar nature had not been recorded elsewhere. Two observations have attracted our attention. In one of them, Zucker and Barnett [1922-23] mention their inability to concentrate the antirachitic activity of plant tissues and of butter by extraction with 95 % alcohol and subsequent saponification of the extract, while this method was successful when applied to cod-liver oil. We are at present repeating Zucker's experiments in order to verify whether and at what stage in his procedure the butter loses its antirachitic potency.

The second paper is by Supplee *et al.* [1931]. These authors, in investigating the antirachitic activity of irradiated milk, extracted the non-saponifiable fraction of milk-fat derived from dry milk or from butter but were unable to activate it antirachitically by ultra-violet irradiation as judged by experiments on rats and chickens, whereas they were able to render equivalent amounts of original natural milk strongly antirachitic under the same conditions.

We wish it to be clearly understood that our results, both with regard to the antirachitic potency of butter and to our inability to concentrate this potency in the non-saponifiable residue, were obtained in experiments on rats, and that our suggestions and conclusions apply to this animal only. The pathological picture brought about in the rat by deprivation of vitamin D combined with a marked alteration in the calcium : phosphorus ratio is by no means identical with rickets in the human being or with similar conditions in other species resulting from the simple withdrawal of vitamin D even in the presence of a correct mineral balance. That caution is necessary in transferring to the human being results obtained on the rat has been sufficiently emphasised by the recent work on the comparative antirachitic potencies of irradiated ergosterol and cod-liver oil.

Numerous experiments in various countries have proved beyond reasonable doubt that butter is antirachitically active when tested on rats. Its value as an antirachitic agent for other species is, however, still very debatable and is yet subject to much controversy.

We are investigating at present the effect of saponification on the antirachitic factor of summer butter and also of butters rendered highly antirachitic either by direct irradiation with ultra-violet light or by the feeding to cows of irradiated yeast or of cod-liver oil. Such butters are sufficiently antirachitic to enable us to test their potency on other species, and we would like to postpone further discussion until these results are available.

SUMMARY.

1. Autumn and winter butters, either saponified in the usual way by boiling for 1 hour on the water-bath with alcoholic KOH, or by heating with alkali for 2 minutes only, lose a large part (over 80 %) of their antirachitic potency as estimated by prophylactic experiments on rats.

2. Under exactly similar conditions, irradiated ergosterol (the International Standard of vitamin D) or cod-liver oil could be subjected to saponification either alone or mixed with butter without loss of potency.

3. The fact that the stability to saponification of the antirachitic factor of cod-liver oil and of irradiated ergosterol is not adversely influenced by the presence of butter speaks against the existence in butter of a specific destructive factor and in favour of a true chemical difference between the antirachitic factor of butter and those of cod-liver oil and irradiated ergosterol.

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