CCCXXV. THE VITAMIN D ACTIVITY OF CACAO SHELL.

I. THE EFFECT OF THE FERMENTING AND DRYING OF CACAO ON THE VITAMIN D POTENCY OF CACAO SHELL.

II. THE ORIGIN OF VITAMIN D IN CACAO SHELL.

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I. THE EFFECT OF THE FERMENTING AND DRYING OF CACAO ON THE VITAMIN D POTENCY OF CACAO SHELL.

THE authors have shown [1934] that one sample of the testa or "shell" of the ordinary dried fermented cacao beans from the Gold Coast had a vitamin D potency of 28 International Units (I.U.) per g. 10 months later another sample of cacao shell was examined by them and found also to contain 28 I.U. per g. As this is quite an exceptional potency for a vegetable product, further investigation was made with the object of determining whether the vitamin D was present in the seed or was produced during the preparation for the market.

The fruit of the cacao tree is a large pod, which contains about 40 cacao seeds or "beans". Cacao shell is the name given to the testa of the bean. It is covered with a layer of mucilaginous pulp which contains about 10 % of reducing sugars and 0.7 % of citric acid. In the approved method of preparation for the market the beans are put in a box and allowed to ferment. After fermentation the beans are dried until their moisture content is below 8 %. They can then be kept without change.

The usual procedure for fermentation on the Gold Coast is to make a conical heap of the beans and cover them with plantain leaves. The method used in the experiment to be described is the more approved method using 3 wooden boxes arranged in steps. The beans are placed in the top box and turned into the lower one after 2 days. They are turned into the lowest box after a further 2 days and are thus fermented 6 days in all. Fermentation of the sugars occurs spontaneously, and the temperature rises, generally reaching 50° in 3-5 days according to the method used. The pulp, by loss of liquid, is diminished in bulk and becomes brown and less mucilaginous. In the later stages acetic and other bacteria develop and, under certain circumstances, moulds also.

During the fermentation of the pulp changes also occur in the interior of the seed. After 2-3 days' fermentation the seed dies. The two folded cotyledons which form the greater part of the seed are purple and, when they die, the purple substance originally in isolated cells becomes distributed throughout them. During the later stages of fermentation and during drying, the colour of

the inside of the bean, that is of the cotyledons, gradually changes from purple to brown owing to the oxidation of the tannins. A little of the tannins, along with the theobromine, also passes from the cotyledons into the shell, which as a result darkens in colour.

The fermented beans are spread out on trays or tables and dried by turning over and over in the sun. At least 6 days are required to dry the beans. The oxidation of the tannins continues as long as the beans are moist. Whilst the beans are damp mould may grow on the shell during the night.

It will thus be seen that the cacao bean normally undergoes two important operations: (1) fermentation in boxes or heaps and (2) drying. Most cacao is fermented and sun-dried, but some cacao is merely sun-dried, and some fermented cacao is artificially dried.

EXPERIMENTAL.

The authors decided to put on tests to determine the effect on the vitamin D content of (1) fermentation and (2) exposure to the sun in drying. The preparation of the cacao was carried out at Cadbury Hall, Kumasi, according to the instructions of one of us (A. W. K.), by Mr F. Nicholas, who is the officer in charge of the Gold Coast Agricultural Department Training Centre. The ordinary Gold Coast cacao belongs to the botanic species *Forastero Amelonado*. Only pods of this species were gathered for the experiment. They were taken from one plot in the plantation. On November 6th, 1934, a sufficient number of pods were collected to provide beans for all the experiments. The beans were extracted from the pods and were well mixed. Four portions of these were taken and submitted to the following treatment:

Sample 1. Beans dried at once in the dark.

Sample 2. Beans dried at once in the sun.

Sample 3. Beans fermented and then dried in the dark.

Sample 4. Beans fermented and then dried in the sun.

Sample 1. The beans *dried in the dark* were taken straight from the pod and dried on wooden trays in a water-oven. The temperature of the oven was maintained near, but did not exceed, 90°. The drying occupied 6 days. The temperature attained by the beans was sufficient to kill them. As a result the purple substance in the cotyledons was diffused. As the temperature in the early stages was not high enough to destroy the oxidase which is present, a certain amount of browning of the interior of the bean took place.

Sample 2. The beans *dried in the sun* were placed at first on a wooden tray and later on palm leaf matting. Owing to the hygroscopic nature of the pulp round the beans, after three weeks the drying was still far from complete. The pulp was thus a suitable medium for the growth of yeasts or mould. As it was feared that the development of mould in the damp mucilage might affect the results, it was decided to wash the beans and thus remove a considerable part of the mucilage. This was done and the beans then dried quickly.

Sample 3. The beans *fermented and dried in the dark* were subjected to the standard fermentation in boxes as described above. Part of them were dried in a water-oven in the same way as those in test 1. The temperature was maintained near, but did not exceed, 90° and drying was complete in 4 days.

Sample 4. The beans *fermented and dried in the sun* were the remaining part of the beans not used in preparing sample 3. They were dried in the normal way at first on wooden trays and later on palm leaf mats. The weather was not favourable and the drying took 22 days.

At the conclusion of the tests the beans were at once placed in tins. They were despatched to England in December 1934. The shells were carefully removed from the beans by hand, and ground to powder in a dark room.

The vitamin D content of the four samples of cacao bean shell was estimated by the method already described [1934]. The fourth sample (fermented and dried in the sun) was found to have a potency of the order of that of the previous samples examined, namely 21 i.u. per g. A preliminary test on samples 1, 2 and 3 showed that their potency was very much less than that of sample 4. The shell was, therefore, incorporated in the diet in order to give the rats a large enough quantity of it. Daily records were made of the amount of food eaten, and the amount of shell eaten was calculated. The diets of the rats for these three tests then consisted of:

	%
Yellow maize	60
Wheat gluten	16
Calcium carbonate	3
Sodium chloride	1
Cacao bean shell	20

The diet in its original form contained 1.37 % Ca and 0.34 % P. An analysis of the cacao bean shell showed that it contained 0.26 % Ca and 0.39 % P. The addition of 20 % shell to the rachitogenic diet therefore reduced the ratio Ca : P from 4 : 1 to about 3.4 : 1. This difference was not great enough to produce healing in the metaphyses of the rats under the conditions of our test [Key and Morgan, 1932].

The results of the tests on the four samples are summarised in Table I.

Table I. *Vitamin D in cacao shell.*

Shell	Treatment of beans	I.U. per g.
Sample 1	Dried in the dark 6 days	0
Sample 2	Dried in the sun more than 21 days; washed	0.6
Sample 3	Fermented and dried in dark 4 days	0
Sample 4	Fermented and dried in sun 22 days	21.0

II. THE ORIGIN OF VITAMIN D IN CACAO SHELL.

In Part I we have described experiments which showed that vitamin D was not present in the shell of cacao beans which had been dried in the dark, whether fermented or unfermented. This indicates that the shell of the cacao bean as it comes fresh from the pod does not contain vitamin D, and yet after exposure to sunlight during drying, vitamin D was present in small amounts in unfermented shell and in very large amounts in fermented shell. Presumably a precursor of vitamin D was present in the shell and was converted into vitamin D by the active rays of the sun. Since the sun-dried, fermented shell was very much more active than the sun-dried unfermented shell, it was decided to carry the comparison further and irradiate both the shells which had been dried in the dark as nearly equally as possible, by exposure to the rays of a quartz mercury-vapour lamp.

EXPERIMENTAL.

Equal weights (56 g.) of the two samples, very finely powdered, were spread evenly in separate galvanised zinc trays of 4 sq. ft. area. These were placed side by side and a quartz mercury-vapour lamp hung 2 ft. 6 in. immediately above the

middle of the space covered by the two trays. The lamp was run at 95 volts, 3.5 amps. In order to ensure equal irradiation of each sample, the trays were turned through an angle of 180° after 8 min. irradiation, and after another 7 min. the powder was collected and respread. The position of the two trays was interchanged. After 8 min. more, the trays were again turned through an angle of 180° and irradiation was continued for 7 more minutes. Thus the total duration of irradiation was 30 min. At the end of this time, there was no visible difference in the appearance of the two powders.

The vitamin D estimation of the two irradiated samples was carried out in the usual way. A little healing was shown in the rats which received the irradiated unfermented shell but as all of them had lost weight during the experiment (an average of 9.5 g.) in spite of having eaten an average of 5.1 g. food per day, the small amount of healing was easily accounted for and had to be disregarded. On the other hand, only one out of 15 rats which received the irradiated fermented shell lost weight, the rest gaining an average of 6.7 g. in the 10 days. A similar experience had been encountered in most of the rats used in testing samples 1 and 3.

The result of the test on the irradiated preparations is given below, together with the previous results for comparison:

Cacao beans used	Vitamin D in cacao shell.		I.U. per g.	
	Dried in the sun. Exposed to sunlight 22 or more days		Dried in the dark	
			Before irradiation	After irradiation
Unfermented	0.6		0	0
Fermented	21.0		0	10.0

It is evident that the process of fermentation has increased enormously the amount of the precursor of vitamin D present in the cacao bean shell.

DISCUSSION.

It will be noticed that the potency of the shell powder irradiated artificially is not as great as that of the cacao shells that had been exposed to sunlight. This could scarcely be expected as both the qualities and the intensities of the two sources of radiation were different. Neither process could be expected to bring about the maximum activation. Moreover, only the outside of the shell of the whole bean was exposed to the sun, whereas the powdered shell exposed to the ultraviolet rays from the lamp was spread so thinly that it is possible that the irradiation was excessive and had resulted in the destruction of part of the vitamin D just formed.

The temperature conditions and period of drying were also different. The solar radiation on the Gold Coast has a mean maximum temperature of 63° and at night the mean minimum temperature is in the region of 23° . The beans were exposed to these conditions for 22 or more days and nights. In the drying oven the temperature continuously approached but did not exceed 90° , the fermented beans being 4 days and the unfermented 6 days in the oven.

In neither case were the temperature conditions severe enough to affect the precursor, and although they might diminish any vitamin D present, they would not result in its complete destruction [Bourdillon *et al.*, 1932]. The absence of either precursor or vitamin D from the unfermented shell dried in the dark indicates that neither is present in the shell originally. Thus the formation of the

precursor of vitamin D is apparently an outcome of the process of fermentation. This process may affect the ergosterol content of the shell or it may not; it is certainly known to favour the growth of micro-organisms (notably yeasts) on the surface of the bean shells. Our theory is that the source of the precursor in the fermented bean shells is the mass of micro-organisms produced during the process of fermentation. This would account not only for the large amount of vitamin D formed on irradiating the fermented shell (either naturally or artificially) but also for the small amount found in the unfermented bean shell dried in the sun; for the periods in the sun were interspersed with cooler night periods during which yeasts could grow.

(a) *The development of the precursor is independent of place or method of fermentation.*

There is some indication that vitamin D is present in the shell of most kinds of fermented sun-dried *Forastero* cacao and that its presence is independent of the country of origin of the cacao. In the preparation of the cacao shell for certain feeding tests [Kon and Henry, 1935] over 1000 tons of roasted beans were de-shelled to obtain a representative sample. The beans consisted mainly of *Forastero* cacao, being a mixture of fermented and, for the most part, sun-dried cacaos from West Africa, the West Indies and South America. The cacaos had been purchased throughout the season. The vitamin D potency obtained by us for this mixture of cacao shells was identical with that obtained for the pure Gold Coast shell previously reported on, namely, 28 I.U. per g. That the precursor is produced by some action which is not peculiar to any one method of fermentation is indicated by the following results obtained from sun-dried cacao from the same producing area, namely, the Gold Coast. The sample previously reported on [1934] was from cacao fermented by the native farmers mainly in heaps, whereas the sample reported on in Part I of this paper was carefully fermented in boxes and regularly mixed. This gave a potency of the same order as, but lower than, that of the native-produced sample.

(b) *Relative potency of vitamin D in shell and interior.*

Manceau and Bigé [1931] have shown that in the seeds which they tested the sterols present in the outer layer (0.02–0.06 %) were slightly greater in amount than in the interior (0.017–0.05 %). Judging from the results of Labbé *et al.* [1929] there is a marked difference in the case of ordinary (presumably fermented) cacao seeds. There are present two sterols, A and B, both of which develop antirachitic properties on irradiation. The B sterol is identical with the ergosterol from brewer's yeast [de Balsac, 1933]. The quantities present are large: 6–8 % of the sterols in the fat from cacao shell and 0.30–0.40 % in the cacao butter from the cotyledons (or nibs). Assuming 54 % of butter in the nibs and 4 % in the shell, the figures would be 0.16–0.21 % sterols in the nib and 0.24–0.32 in the shell. The difference in vitamin D found by the authors [1934] is yet more marked; using Gold Coast cacao the nibs gave 1 I.U. per g. as compared with 28 I.U. for cacao shell.

Figures for the potency of vitamin D have been published for comparatively few foodstuffs. Amongst animal products, save for liver oils, it is rare to find 1 I.U. per g. exceeded, and in vegetable products the amounts recorded are so small that the cacao bean is unique in its vitamin D content both in the interior and in the shell. Whilst the wide differences between our vitamin D figures for nib and shell might be explained as due to the exposure of the shell to the sun,

the difference between the amounts of sterols in nib and shell needs further explanation, as does the striking difference in the vitamin D potencies of the shell of fermented and unfermented beans.

(c) *The production of the precursor of vitamin D.*

Below we consider some possible causes of the production of the precursor in the shell during fermentation.

(1) *Changes due to incipient germination and death of the cacao seed.* In the early stages of fermentation the temperature rises and the conditions are favourable to incipient germination. Little visible change occurs because within 60 hours from the beginning of the fermentation the germ is killed. Soon after the cotyledons also die. Little is known regarding the incipient germination but the changes that follow the death of the seed have received attention.

Tests for lipase have been made. Brill [1915] found none in either fermented or unfermented beans and Ciferri [1931] found lipase only in unfermented beans. The absence of lipase from fermented beans is in accord with practical experience of the good keeping properties of both cacao beans and cacao butter. During fermentation, after the death of the seed, certain substances pass from the nib to the shell, e.g. theobromine [Knapp and Wadsworth, 1924] and tannins [Whympers, 1933]. The fat obtained from the shell possesses a high percentage of the vitamin D activity of the shell [Kon and Henry, 1935] so that changes in the fat percentage are of interest. In our samples the fat percentages are lower after fermentation.

Analyses of the shells.

Sample	Shell on bean %	Moisture in shell %	Fat in shell.
			Light petroleum extract %
Unfermented (dried in dark)	12.9	3.3	1.4
Fermented (dried in dark)	10.8	6.3	1.1
Unfermented (dried in sun and washed)	6.5	8.8	1.7
Fermented (dried in sun)	12.9	8.3	1.2

As ergosterol is considered to be primarily a product of carbohydrate metabolism, the possibility of shell acquiring this from the cotyledons is small.

(2) *Changes in the pulp which may produce the precursor in the shell.* The unfermented pulp contains about 10% reducing sugars, the fermented pulp naturally contains less. Mere exposure of fruits which are rich in natural carbohydrate, e.g. dates, apricots, to the tropical sun, does not produce vitamin D [Lecoq, 1932].

The simplest explanation of the production of vitamin D in cacao shell is that it is due to yeast, which grows in the pulp on the outside of the bean during fermentation and is irradiated by the sun during drying.

Various investigators [Ciferri, 1931] have shown that in the early stages of fermentation *Endomyces anomalus* and members of the *Saccharomyces apiculatus* group (e.g. *Klockeria cacaicola*) are the most common organisms present, although *Euturulopsis theobromae* is also to be found. The latter yeast is fairly common throughout fermentation, but in the later stages the most abundant are *Torulopsis Lilienfeld-Toalii*, *Saccharomyces ellipsoideus* and *Schizosaccharomyces Bussei*. The last two survive both fermentation and drying and are found on practically all kinds of commercial cacao beans [Lilienfeld-Toal, 1927]. From fermenting cacao on the Gold Coast, Dade has isolated a new *Mycotoruloides*, a *Klockeria*, a *Hansenula* and a *Mycoderma* [Knapp, 1935]. It may be well to com-

plete the list of micro-organisms which may be found on the shell. As the fermentation continues acetic acid is formed to a greater or less extent, and Eckmann [1928] has shown that on cacao beans from all countries acetic bacteria can be found, e.g. *B. xylinum*, *B. xylinoides*, *B. orleanense* and *B. ascendens*.

In well conducted fermentations the beans are turned at intervals from one box to another to mix and aerate the mass. On the Gold Coast the farmer sometimes fails to give his heap a single turning or mixing. In this case by the fifth day certain moulds will be present, particularly the thermophiles, *Aspergillus fumigatus* and *Mucor Buntingii* [Bunting, 1928]. During drying certain other moulds may grow on the shell of the bean: aspergilli, mucors or penicillia, particularly *A. flavus*, *A. tamarii*, *A. niger*, *A. glaucus* and *A. sydowi*. If drying is continued these will cease to grow and will become no longer readily visible to the eye.

Of the micro-organisms concerned in cacao fermentation, the acetic bacteria can presumably be ignored as a source of ergosterol. Certain specific moulds have been shown to synthesise ergosterol. This is found in the dried mycelia [Birkinshaw *et al.*, 1931] and on irradiation moulds acquire anti-rachitic properties [Preuss *et al.*, 1931].

The appearance of mould on cacao during drying depends on the weather conditions and on the care taken. Given bad conditions or carelessness it may appear on the shell of either fermented or unfermented beans. The moulds recorded in both cases are for the most part of the same species. The irregular incidence of mould would not account for the regularity of the vitamin D found in the shell of fermented beans. We conclude that mould is a relatively unimportant source of the precursor of vitamin D in cacao shell.

The presence of yeast explains the observed facts better. If cacao is put in a box and allowed to ferment, the pulp shows great numbers of yeast cells after one day and the numbers are maintained for several days. Ciferri gives the number of mould spores on healthy fermented cacao as being about 1000 per bean; according to Dade the number of yeast cells during fermentation is of the order of 2000 millions per bean. When unfermented beans are spread in a layer to dry, yeast develops on the first day, but later conditions are not continuously favourable to its growth. In our experiments, as sometimes occurs in normal practice to facilitate sun drying, the unfermented beans were washed. This removed much of the pulp and with it much of the yeast present.

Yeast cells occur in quantity on the normal dried fermented bean, often as large colonies. They are also present in quantity on beans which have not been deliberately fermented but have been dried in the sun. They cannot be washed off and counted as they are distributed throughout the dried pulp layer of the shell. An attempt was made in the experimental samples to get some idea of the quantity of yeast cells by soaking and scraping off the surface of the shell. The unfermented beans whether dried in the dark or dried in the sun (and washed) showed very few yeast cells. The fermented beans in both cases showed a vast number, the greater number being present on the beans dried in the sun.

The capacity of the tropical variety of *S. ellipsoideus*, or of other cacao yeasts, for elaborating ergosterol is not known, but ordinary *S. ellipsoideus* in the dry state contains an average amount of ergosterol for a yeast, namely 0.6% [Bills *et al.*, 1930].

One of us [Coward, 1933] has reported the anti-rachitic activation of yeast by exposure to strong sunlight. The thin layer of yeast on the surface of the shell is well placed for efficient irradiation during drying.

Steenbock *et al.* [1930] and Kon [1931] by irradiating distiller's yeast have

raised its anti-rachitic potency to approximately 75 times that of a good cod-liver oil, whilst the yeast produced by Steenbock *et al.* [1930] for feeding cattle had a potency of 2700 I.U. vitamin D per g. Taking the latter potency as one not difficult to attain, about 1% of irradiated yeast on the shell of the cacao bean would give the figures 21 and 28 which we obtained for the fermented cacao. In the case of the unfermented beans dried in the sun the limited fermentation and the washing away of a good part of the pulp resulted in a figure of only 0.6 units. The conditions for the drying of beans taken straight from the pod and dried in the dark did not permit of the growing of yeast and hence with these we found no vitamin D after irradiation.

The growth of yeast in the pulp, combined with the exposure to the sun, satisfactorily accounts for the vitamin D in shell.

Both the vitamin D and the sterols in the cotyledons are unusually high. The manner of their production requires further investigation.

SUMMARY.

1. It is improbable that either vitamin D or ergosterol is present in the fresh shell of the cacao bean.

2. During fermentation, yeast containing ergosterol develops in the pulp on the shell.

3. During drying in the tropical sun the ergosterol is converted into vitamin D.

4. Hence the order of vitamin D potency of the shell of the cacao bean is: (1) artificially dried—absent; (2) not deliberately fermented but slightly fermented during sun-drying—fairly high, approaching the potency of dairy butter; (3) fermented and sun-dried—very high, twenty or thirty times the potency of dairy butter.

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REFERENCES.

- de Balsac (1933). *Acad. Agric. France*, **19**, 862.
Bills, Massengale and Prickett (1930). *J. Biol. Chem.* **87**, 259.
Birkinshaw, Callow and Fischmann (1931). *Biochem. J.* **25**, 1977.
Bourdillon, Bruce and Webster (1932). *Biochem. J.* **26**, 522.
Brill (1915). *Philippine J. Sci.* **10** (A), 123.
Bunting (1928). *Dept. Agric. Gold Coast Year Book*, **44**.
Ciferri (1931). *J. Dep. Agric. Porto Rico*, **15**, 223.
Coward (1933). *Lancet*, ii, 930.
Eckmann (1928). Dissertation, Kiel.
Key and Morgan (1932). *Biochem. J.* **26**, 196.
Knapp (1935). *Bull. Imp. Inst.* **33**, 31.
— and Coward (1934). *Analyst*, **59**, 474.
— and Wadsworth (1924). *J. Sci. Chem. Ind.* **43**, 124 T.
Kon (1931). *Lancet*, ii, 579.
— and Henry (1935). *Biochem. J.* **29**, 2051.
Labbé, de Balsac and Lerat (1929). *Compt. Rend. Acad. Sci.* **189**, 864.
Lecoq (1932). *Compt. Rend. Soc. Biol.* **111**, 293.
Lilienfeld-Toal (1927). *B. Tropenpflanzer*, **30**, No. 2.
Manceau and Bigé (1931). *Compt. Rend. Soc. Biol.* **107**, 635.
Preuss, Petersen, Steenbock and Fred (1931). *J. Biol. Chem.* **90**, 369.
Steenbock, Hart, Hanning and Humphrey (1930). *J. Biol. Chem.* **88**, 197.
Whymper (1933). *Chim. Ind.* **30**, 507.