The availability of bound nicotinic acid to the rat

3.* The effect of boiling maize in water

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The bound form of nicotinic acid in maize is fairly stable in weakly acid solutions, though in a weak alkali it decomposes readily to yield nicotinic acid in its free form (Chaudhuri & Kodicek, 1950; Kodicek, 1951). Treatment of maize with lime-water, as used in the preparation of *tortilla* in Mexico, releases nicotinic acid from its bound form and enables it to cure nicotinic-acid deficient rats, as originally observed by Laguna & Carpenter (1951) and confirmed by others (for references see Kodicek, Braude, Kon & Mitchell, 1959). Deficient pigs are also cured by such a *tortilla* preparation (Kodicek *et al.* 1959).

Most of the workers in this field accept the view that the beneficial effect of alkali-treated maize on nicotinic-acid deficiency is due to liberation of the free from the bound form of nicotinic acid, as discussed in previous papers (Kodicek & Wilson, 1959; Kodicek, 1960). This view may explain, in part, the low incidence of pellagra in populations consuming *tortilla*. A possible coincidental consumption of other foods or beverages rich in available nicotinic acid may be a further factor (Krehl, 1949; Bressani, Marcucci, Robles & Scrimshaw, 1954; Pearson, Stempfel, Valenzuela, Utley & Darby, 1957; Kodicek & Wilson, 1959; Squibb, Braham, Arroyave & Scrimshaw, 1959).

However, Pearson *et al.* (1957) have recently reported that boiling maize for 4 h with water alone releases nicotinic acid from its bound form. This apparent instability of the bound form in aqueous solutions would tend to militate against the importance of lime-water treatment as an efficient pellagra-preventive measure. Harper, Punekar & Elvehjem (1958) were unable to confirm this finding; they did not obtain any cure of nicotinic acid-deficient rats, nor could they prevent the development of deficiency in rats by maize that had been boiled in water for 1·5 and 4 h, whereas maize treated with lime-water was invariably effective.

In view of the importance of this question in connexion with the pellagra problem, we have re-investigated the effect of boiling maize in water on the release of nicotinic

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acid from its bound form.* Part of the work was carried out at the Dunn Nutritional Laboratory and part at the Rowett Research Institute, referred to below as D.N.L. and R.R.I., respectively.

EXPERIMENTAL

Methods

Estimation of nicotinic acid. Total nicotinic acid was determined chemically and microbiologically as described previously (Kodicek & Wilson, 1959). The free and the bound nicotinic acid were estimated semi-quantitatively by the response of *Lactobacillus casei* to the two forms and determined by quantitative paper chromatography with 80% methanol extracts, as described by Kodicek & Wilson (1959).

Feeding trials with rats

Expts 1 and 2 (D.N.L. series). These experiments were similar to those of Kodicek & Wilson (1959). Hooded Lister male weanling rats (50–60 g) of the D.N.L. colony were allotted at random, four to each of ten experimental treatments (see Table 4). They were housed in individual cages, with raised wire floors, and for a preliminary period of 17 days all received the same depleting diet of Harris & Kodicek (1950) containing 3·5% casein and 40% maize meal as the sole sources of protein. At the end of this period the rats ceased gaining or had lost weight. They were then transferred to basal diet 1 (Table 1), which contained one of five maize preparations (A–E, to be

Table 1. *Percentage composition of basal diets*

Constituent	Diet 1 (D.N.L.)	Diet 2 (R.R.I.)	Diet 3 (R.R.I.)
Maize preparation (A–E)	40·0	40·0*	40·0*
$Ca_3(PO_4)_2$	—	2·0	2·0
Casein, 'vitamin-free' (Genatosan Ltd)	3·5	3·5	7·0
Sucrose	51·4	48·9	45·35
Cottonseed oil	2·0	—	—
Linseed oil	—	2·0	2·0
Cod-liver oil	—	1·0	1·0
L-Cystine	0·1	0·1	0·15
Minerals†	3·0	2·5	2·5
Vitamins†	+	+	+

* $Ca_3(PO_4)_2$ was already in the maize preparation E, of which 42% provided both constituents.
† In diet 1, the mineral and vitamin supplements (without nicotinic acid) were as used by Kodicek & Carpenter (1950); in diets 2 and 3 the supplements were those used by Laguna & Carpenter (1951).

described below). As shown in Table 4, for groups 1, 2, 5 and 6, 40% of the untreated maize (A or C) was included; for groups 3 and 4, 40% of the 1 h boiled maize (B), for groups 7 and 8, 40% of the 5 h boiled maize (D) and for groups 9 and 10, 40% of the maize treated with lime-water (E) was added to the diet. Supplements of nicotinic acid, 10 mg/kg, were given to rats in groups 2, 4, 6, 8 and 10. These variants of diet 1 were all offered *ad lib.* as dry powders for a further 28 days, during which the gain in weight was recorded twice weekly and the food consumption daily.

* Dr A. E. Harper, to whom certain of our findings were made available some time ago, has kindly referred to them in a recent paper (Harper *et al.* 1958).

Expt 3 (*R.R.I. series*). In this experiment, weanling hooded Lister rats of the R.R.I. colony were allotted to the experimental treatments at random without a preceding period of deficiency. The experimental diets were given for 24 days; they were all of the basic type shown as diet 2 in Table 1. It differed slightly from diet 1 in the type of oil used and the vitamin and mineral supplements; like diet 1, it contained 3·5% casein.

The maize preparations used in this basal diet are set out in Table 5. For groups 15 and 16, 42% of maize treated with lime-water was included. This preparation (E) contained all the dried liquors from the cooking process (rich in $Ca_3(PO_4)_2$ from neutralization of the lime-water), and it was balanced for the other groups (11–14) by including 40% of either untreated maize (C) or 5 h boiled maize (D), together with 2% $Ca_3(PO_4)_2$. (No such allowance had been made in Expts 1 and 2, in which each diet had 1·3% $Ca_3(PO_4)_2$ contributed by the mineral mixture.)

The general conditions of caging and feeding the rats were the same as for Expts 1 and 2, but the experiment lasted, as mentioned before, only 24 days, and only the total food consumption of the rats on each treatment was recorded.

Eight rats were allotted to each of the groups 11, 13 and 15, which received no supplement of nicotinic acid, and six rats to each of groups 12, 14 and 16 for each of which 30 mg nicotinic acid/kg were added to the diet. In this experiment, and in the next one, the rats allocated to each group were males and females in equal numbers.

Expt 4 (*R.R.I. series*). The conditions were the same as for Expt 3 (without a preliminary deficiency period), except that the basal diet used was diet 3 (Table 1), which contained an extra 3·5% casein (at the expense of sucrose) as compared with diet 2, and also that the trial continued for 31 days. Here again eight rats were used for each of the groups 17, 19 and 21, which did not have a nicotinic-acid supplement, and six rats each for groups 18, 20 and 22, which did.

Materials

Samples A, B, C, D and E (described below) were used in the D.N.L. series of experiments; in the R.R.I. series only samples C, D and E were used. All were ground to a meal before being used for analysis or feeding trials.

Prepared at D.N.L.

Untreated maize (*A*). It was a commercial sample of Plate Argentinian yellow maize containing 8·5% crude protein.

1 *h boiled maize* (*B*). It was prepared from maize A in the same way as *tortilla* by a Mexican recipe (Kodicek & Wilson, 1959), except that tap water (pH 7·2) was used in place of lime-water. The procedure consisted in soaking kernels of maize overnight in water, then boiling for 20 min, decanting the supernatant liquid, heating the residue with tap water (3 parts to 1 part original maize) for 1 h at 80° and allowing the mixture to stand overnight. A small amount of water was then mixed in and the supernatant liquid decanted; the cooked maize was mashed and baked on a girdle into flat cakes which were dried at 70°.

Prepared at R.R.I.

Untreated maize (*C*). It was a commercial sample of yellow maize of unknown origin, containing 8·9% crude protein.

5 h boiled maize (*D*). It was prepared by simmering 4·25 kg of maize C in 10 l. tap water (pH 6·6) for 5 h in an open pan with additions of water to keep the volume constant. The preparation was then dried as for E.

Maize treated with 1% lime-water (*E*). It was prepared from whole maize C by stirring with 1% lime-water (3 parts to 1 part maize) at 80° for 50 min, leaving it to steep overnight and then adjusting the pH to 7 with orthophosphoric acid. The solids were then minced and dried in a hot-air oven at 80° with gradual addition of the liquors, as already described (Laguna & Carpenter, 1951).

RESULTS

Nicotinic acid in maize preparations

The results of the analyses for free, bound and total nicotinic acid are set out in Table 2. The two samples of untreated maize, A and C, contained about 98% of their nicotinic acid in the bound form. At the opposite extreme the sample E, which was prepared by cooking with lime-water, had its nicotinic acid entirely in the free form.

Table 2. *Contents of nicotinic acid (free and bound) and crude protein in constituents of the diets*

Constituent	Nicotinic acid (μg/g)*			Crude protein (%)
	Free	Bound	Total	
Maize preparation:				
A, untreated Argentinian maize (D.N.L.)	0·3	18·0	18·3	8·5
B, 1 h boiled maize (D.N.L.)	0·2	10·5	10·7	8·5
C, untreated maize (R.R.I.)	0·4	25·3	25·7	8·9
D, 5 h boiled maize (R.R.I.)	3·8	20·0	23·8	8·9
E, maize treated with 1% lime-water (R.R.I.)	24·6	0	24·6	8·9
Casein, 'vitamin-free' (Genatosan Ltd)	3·5	0	3·5	91·5

* Values represent means of chemical and microbiological estimations. Paper-chromatographic analyses showed that about 2% of the nicotinic acid in untreated maize and in the 1 h boiled maize was in the free form derived from maize germ. In 5 h boiled maize, only a further 14% had been released from the bound form; in maize treated with lime-water all the nicotinic acid was free.

Sample B, prepared by boiling for 1 h with tap water, had no more free nicotinic acid than the original maize, and sample D, similarly prepared but with 5 h boiling, had only 14% of its nicotinic acid released from the bound form, apart from that present in the original maize (about 2%). The losses in the total nicotinic-acid content of B and D were approximately 40 and 7%, respectively; the difference may have been due to the discarding of supernatant liquors in the preparation of B.

The amount of free nicotinic acid released from the bound form in maize after 5 h boiling in tap water tallied well with the findings on a purified preparation of bound nicotinic acid from wheat bran (Table 3). It was made by the procedure of Chaudhuri

& Kodicek (1950), and contained 37 mg nicotinic acid/g, in bound form. When this preparation, 10 mg dissolved in 1 ml tap water, was boiled, there was a release of only 1% after 1 h, and even after boiling for 4 h only 15% of the nicotinic acid appeared in the free form. The former finding is in agreement with the results obtained for 1 h boiled maize in the D.N.L. series; then no free nicotinic acid was liberated. In contrast, boiling the purified preparation with 0·1% lime-water released all the nicotinic acid from the bound form within the first 10 min.

Table 3. *Percentage release of nicotinic acid from a purified preparation* (10 *mg*) *of bound nicotinic acid* by boiling in either* 1 *ml tap water* (*pH* 7·2) *or* 1 *ml of* 0·1% (*w*/*v*) *lime-water*

	Release after					
	0·2 h	1 h	2 h	3 h	4 h	8 h†
In tap water	—	1	3	6	15	30
In lime-water	100	100	100	—	—	—

* Made by the procedure of Chaudhuri & Kodicek (1950), somewhat modified. It contained 37 mg bound nicotinic acid/g. Free nicotinic acid was estimated by paper chromatography (Reddi & Kodicek, 1953).

† Followed by a further period of 16 h standing at room temperature.

Feeding trials with rats

Table 4 shows the mean values for gain in weight of the rats during the experiments in the D.N.L. series, together with their intake of food and nicotinic acid. It will be seen that in Expts 1 and 2, in which a diet containing 3·5% casein was given, rats in

Table 4. *Expts* 1 *and* 2 (*D.N.L.*). *Mean values with their standard errors for gain in weight and food intake and mean values for intake of free, bound and total nicotinic acid of rats given the different maize preparations with or without added nicotinic acid after a preliminary deficiency period*

Expt no.	Group no.	Maize preparation used in diet 1 (see Table 2)	Supplement of nicotinic acid (mg/kg diet)	Gain in weight (g/week)	Food intake (g/day)	Intake of nicotinic acid (μg/day)		
						Free*	Bound	Total
1	1	A (untreated)	—	−2·7 ± 1·4	6·2 ± 0·5	1	45	46
	2		10	21·8 ± 1·6	13·7 ± 0·4	140	99	239
	3	B (1 h boiled)	—	−3·8 ± 0·5	5·3 ± 0·3	1	22	23
	4		10	26·0 ± 4·0	15·0 ± 1·5	153	63	216
2	5	C (untreated)	—	0·4 ± 2·8	6·6 ± 0·8	2	66	68
	6		10	22·3 ± 0·7	14·1 ± 0·7	145	143	288
	7	D (5 h boiled)	—	6·8 ± 2·0	8·5 ± 0·9	14	68	82
	8		10	21·9 ± 2·9	14·2 ± 1·2	165	114	279
	9	E (treated with lime-water)	—	22·3 ± 2·7	15·2 ± 1·1	151	0	151
	10		10	25·0 ± 1·0	16·5 ± 0·6	329	0	329

* Derived from casein, maize germ and maize after treatment, and from added nicotinic acid.

groups serving as deficient controls (groups 1 and 5) gained little or lost weight; one rat died on the 19th day in group 1 and two on the 23rd day in group 5. The positive control animals in groups 2 and 6, given a nicotinic-acid supplement, grew well at a rate of about 22 g/week.

The rats in group 3, given the diet with the maize boiled for 1 h in tap water (B), remained deficient, and one died on the 21st day of the experiment proper. Supplementation of this diet with nicotinic acid (group 4) cured the deficiency. The rats in group 7, given the diet with maize boiled for 5 h in tap water (D), appeared to show a slightly better gain in weight, 6·8 g/week, than the deficient controls in group 5, but the difference was not quite significant ($P \sim 0{\cdot}1$, t test). Supplementation of diets containing the 5 h boiled maize (D) with nicotinic acid resulted in complete cure of the deficiency state. The rats in group 9 given a diet with maize treated with 1% lime-water (E) also responded well and grew as fast as either the rats of group 10, which had received the same diet supplemented with nicotinic acid, or the positive control rats (group 6).

Table 5. *Expts 3 and 4 (R.R.I.). Mean values with their standard errors for gain in weight and mean values for food intake and intake of free, bound and total nicotinic acid of rats given the different maize preparations with or without added nicotinic acid, without a preliminary deficiency period*

Expt no.	Group no.	Diet no.	Maize preparation used	Supplement of nicotinic acid (mg/kg diet)	Gain in weight (g/week)	Food intake (g/day)	Intake of nicotinic acid (μg/day) Free*	Bound	Total
3	11	2 (3·5% casein)	C (untreated)	—	1·6 ± 0·8	6·3	1	65	66
	12			30	10·9 ± 1·3	9·0	273	91	364
	13		D (5 h boiled)	—	4·5 ± 0·5	9·0	15	72	87
	14			30	6·8 ± 0·4	7·5	237	60	297
	15		E (treated with lime-water)	—	6·7 ± 0·8	8·7	91	0	91
	16			30	7·9 ± 0·7	9·5	384	0	384
4	17	3 (7% casein)	C (untreated)	—	20·5 ± 1·4	10·5	4	106	110
	18			30	26·8 ± 2·5	13·0	395	132	527
	19		D (5 h boiled)	—	23·1 ± 1·5	10·5	19	84	103
	20			30	25·6 ± 2·0	12·7	403	102	505
	21		E (treated with lime-water)	—	24·6 ± 1·6	12·1	128	0	128
	22			30	26·0 ± 2·6	11·7	475	0	475

* Derived from casein, maize germ and maize after treatment, and from added nicotinic acid.

The performance of the rats in Expts 1 and 2 tallied well with their intake of free, available nicotinic acid resulting either from liberation of nicotinic acid or from supplements of nicotinic acid (see Table 4).

Table 5 shows the performance of rats and their daily intake of food and nicotinic acid in Expts 3 and 4 of the R.R.I. series. The management of the rats, duration and design of the experiments were all different from those of the D.N.L. series; the results were somewhat different also, but the overall trend was the same. As mentioned

before, these experiments were designed as preventive tests, and the animals were therefore in good health, not deficient as in Expts 1 and 2.

In Expt 3, with diets containing 3·5% casein, rats in group 13 given a diet with 5 h boiled maize (D) had a significantly better weight gain than those in group 11 receiving the untreated maize (C) ($P < 0.01$, t test). The performance of rats in group 13 was, however, significantly worse than in group 15 given the diet with the maize treated with lime-water (E) ($0.02 < P < 0.05$, t test). It will be noted that all the groups of rats receiving supplements of nicotinic acid (groups 12, 14, 16) gained weight more slowly than those at Cambridge.

The response in weight gain agreed well with the intake of free, but not with that of total, nicotinic acid; thus rats in group 11 consumed daily 60 μg total nicotinic acid, almost all in the bound form, and became deficient, whereas rats in group 15 with a daily intake of 91 μg total nicotinic acid, all in the free form, grew well on the diet containing maize treated with lime-water. Rats in group 13, given a diet with 5 h boiled maize (D), ingested daily 15 μg of free nicotinic acid, and their growth performance was slightly superior to that of the deficient controls; however, it was not as good as that of rats in group 15 receiving maize treated with lime-water (E), although the amounts of total nicotinic acid consumed were similar, i.e. 87 and 91 μg, respectively.

In Expt 4, the basic diet contained 7% casein, and it will be seen from Table 5 that the rats in the deficient control group 17 showed only a small retardation of growth. The absolute values for weight gain were higher than those produced by diets of lower protein content, but the percentage responses were smaller, reducing the differences in weight gain between groups of rats consuming various amounts of available nicotinic acid. The difference in weight gain between the rats in group 17 and group 18 supplemented with nicotinic acid was just significant ($P \sim 0.05$, t test), but the other estimates of differences were not significant ($P > 0.1$, t test). Statistical treatment of values for food intake was not possible, since only the total consumptions were measured.

DISCUSSION

Our object was to investigate whether or not methods of cooking maize that do not include any alkali treatment would, under our experimental conditions, release the pellagra-preventive activity for rats that is released when maize is boiled with either lime-water or NaOH. If such a change were to be observed, it would weaken the hypothesis that the prime cause of the improvement with alkali treatment is the liberation of nicotinic acid from its bound form, since boiling at neutral pH gives only a small slow release of the vitamin.

For the first trial a sample of maize was cooked by exactly the process we have used for making *tortilla* (Kodicek & Wilson, 1959) but with ordinary tap water instead of lime-water. This product (B), which had been boiled in water for 1 h and steeped for 40 h, gave a response no different from that to untreated maize in the usual type of D.N.L. assay with a diet containing 3·5% casein. A similar experiment with the same result has now also been reported by Harper *et al.* (1958).

The cooking process described in the paper by Pearson *et al.* (1957), in which it was originally suggested that maize could be improved by boiling in water alone, included a 5 h period of boiling. For our remaining trials a product made with 5 h boiling (D) was used. In both the second and third feeding experiments, rats given this material grew slightly better than those given the corresponding uncooked maize, but the response was not equal to that obtained with material cooked in lime-water for a much shorter period.

This partial response with the material boiled in tap water for 5 h was not in conflict with the chemical analyses. The usual analytical procedure showed that about 14% of the bound nicotinic acid was liberated during the process. This finding in turn was in agreement with the results of a test (shown in Table 2) in which a purified preparation of bound nicotinic acid had also been hydrolyzed to the extent of 15% after 4 h of boiling. The same purified preparation, when dissolved in lime-water, had been hydrolyzed completely after boiling for 10 min. (In maize, for complete liberation of nicotinic acid from its bound form, a longer treatment with lime-water is necessary. One could not expect the same speed of reaction with maize itself because of the time taken for the lime-water to penetrate the grain.)

Another difference between our experiments and those of Pearson *et al.* (1957) was that the latter used basic diets of a higher casein content. It has been the general experience that under these conditions growth depression with untreated maize, without supplementary nicotinic acid, is smaller and less regular. It was so in our own fourth experiment, in which the casein content of the basic diet was raised to 7%, the growth of rats with untreated maize being about 75% of that with a nicotinic-acid supplement. The apparent differences due to various treatments were small and statistically not significant, though they showed the same trend as the low-casein experiments. Failure to obtain clear-cut deficiency under such conditions has been reported on several occasions (Henderson, Deodhar, Krehl & Elvehjem, 1947; Kodicek, Carpenter & Harris, 1947; Krehl, Carvalho & Cowgill, 1947). Presumably the marginal situation with this diet could mean that even a small amount of free nicotinic acid could tip the balance.

Yet a third difference between our own experiments and those of Pearson *et al.* (1957) was that the latter dried their material at a higher temperature, i.e. 120°. We have made no study of the effect of this temperature on the nutritive value of maize products; it is possible that such drying can cause changes accounting in part for the differences between their results and those of Harper *et al.* (1958) and of ourselves.

Pearson *et al.* (1957) have reported that when 10 g of maize were suspended in 1 l. distilled water, much of the nicotinic acid present was released from its bound form. This finding does not agree with our observations on maize or on a purified preparation of bound nicotinic acid, either now presented or obtained in the course of purification of bound nicotinic acid (Chaudhuri & Kodicek, 1950; Kodicek & Wilson, unpublished results). If the release of nicotinic acid from its bound form occurred to the extent found by Pearson *et al.* (1957), we should have observed it during purification procedures and incurred large losses of the bound form. Further, no marked liberation of nicotinic acid in maize products was observed, provided the pH did not

exceed 7·0–7·2; when 1 part maize has been suspended in 3 parts water, the pH has usually been of the order of 6·2–6·6, owing to the natural acidity of the maize (Kodicek & Wilson, 1959). The release of nicotinic acid in water must be small, since the bound form in beer, derived from barley, survives the whole process of brewing and the nicotinic acid in beer is still unavailable (Kodicek & Wilson, 1959). That soaking of maize in water for long periods has no effect on the bound nicotinic acid can also be seen from the results of nicotinic-acid analysis of a native maize-flour preparation from Nyasaland, called *ufa*. This product is made from maize by an involved process that includes soaking broken, dehusked, degermed maize in water for at least 2 days and sometimes for as long as 2–3 weeks. The nicotinic-acid content of *ufa* was found to be 4·6 μg/g, all apparently in the bound form (Kodicek, unpublished).

From our results we conclude that maize products prepared in a way involving alkali treatment support rapid growth in rats under conditions in which similar products prepared by boiling without alkali treatment do not. Further, these results were predicted from the chemical analysis of the products for free and bound nicotinic acid, on the hypothesis that only the former is of biological value. There has been no evidence that treatment with lime-water can cause such a marked difference in the properties of maize by any other mechanism.

SUMMARY

1. In curative or preventive experiments with weanling rats the effect of boiling maize in tap water or in 1% lime-water was tested to ascertain the degree of liberation of nicotinic acid from its bound form.

2. The amount of bound nicotinic acid liberated by the various treatments was also determined chromatographically and microbiologically.

3. Boiling maize in tap water for 1 h, combined with soaking for over 40 h, did not result in any detectable release of nicotinic acid from its bound form; the resulting material did not cure rats suffering from nicotinic-acid deficiency.

4. Boiling maize for 5 h, combined with previous soaking for over 20 h, released only about 14% of nicotinic acid from its bound form, and the resulting maize preparation had only a small curative or preventive effect, corresponding to the small amount of nicotinic acid liberated.

5. Maize treated with 1% lime-water had all its nicotinic acid in the free form and completely protected rats from, or cured them of, nicotinic-acid deficiency.

6. A purified preparation of bound nicotinic acid, containing 37 mg nicotinic acid/g, all in the bound form, was boiled in tap water for over 8 h. In the 1st h only 1% of the nicotinic acid was released, and even after 4 h boiling, only 15% was liberated. On the other hand, boiling the preparation in 0·1% lime-water released all the nicotinic acid within 10 min.

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