NEPHROCALCINOSIS IN THE WHITE RAT

I. THE NEPHROCALCINOGENIC DIET OF GILBERT AND GILLMAN[1]

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Gilbert and Gillman[1] found that the supplementation of maize meal with soy-bean meal alone was not sufficient to support optimal growth and ensure survival in rats. To overcome the inadequacies of the soya-maize meal mixture, these authors found it necessary to add sources of fat- and water-soluble vitamins as well as a Steenbock 40 mineral mixture.[2] It was observed, however, that the additions of the mineral mixture to the soya-maize meal dietary setting not only seriously interfered with reproduction, but also precipitated kidney lesions (nephrocalcinosis).

In a National Nutrition Research Institute publication[3] on the supplementation of cereal diets it is recommended that further work should be done on the development of suitable soya foods to combat malnutrition. In this respect, the findings of Gilbert and Gillman[1] are of great importance and warrant further investigation. Wise and Kark[4] reported that kidney and bladder stones very seldom occur among our indigenous population groups, whose staple diet is mainly maize. Curative and preventive measures employed to combat malnutrition, such as those which entail the addition to the maize diet of so-called 'supplements', therefore need very careful scrutiny, to ensure that the incidence of nephrocalcinosis remains at its present low level.

Unfortunately the information published by Gilbert and Gillman[1] does not reveal the true cause of the kidney lesions observed. It is not possible to decide on the basis of the data presented whether the stones were formed as a result of the inclusion of the soy-beans or whether stone formation was due to the influence of those dietary factors known to cause kidney stones, e.g. (i) high magnesium, high phosphorus and low calcium intakes;[5] (ii) low magnesium, low phosphorus and high calcium intakes;[5] (iii) low magnesium, high phosphorus and high calcium intakes;[5] (iv) excessive intake of vitamin D;[6] (v) excessive intake of vitamin C;[7] (vi) inadequate intake of pyridoxine;[8,9] and (vii) inadequate intake of vitamin A.[10]

In view of the practical importance of the problem and the lack of clear-cut information regarding the mechanisms involved, it was decided to conduct a series of experiments in an attempt to reveal the cause of the nephrocalcinosis observed.

In the present paper the results are reported of a comparison of the nephrocalcinogenic effects of the diet used

TABLE I. CONSTITUENTS OF EXPERIMENTAL DIETS

Constituent	*Weight in G/100 G, of air-dry diet*							
	Diet I	*Diet* II	*Diet* III	*Diet* IV	*Diet* V	*Diet* VI	*Diet* VII	*Diet* VIII
Maize meal porridge	69·5	69·5	69·5	69·5	69·5	69·5	72·0	69·3
Soy-bean meal (cooked, hexane-extracted)	18·5	18·5	18·5	18·5	18·5	18·5	19·2	18·5
Food yeast	4·6	4·6	4·6	4·6	4·6	4·6	4·8	—
Vitamin mixture	—	—	—	—	—	—	—	2·0
Cod-liver oil	2·0	2·0	2·0	2·0	2·0	2·0	2·0	2·0
Wheat-germ oil	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Dextrin	1·9	—	4·6	3·5	4·0	2·9	—	—
Steenbock 40 mineral mixture	3·0	3·0	—	—	—	—	3·0	3·0
$CaCO_3$	—	1·9	—	1·37	—	1·37	—	—
KH_2PO_4	—	—	—	—	0·57	0·57	—	—
NaCl	—	—	0·3	0·3	0·3	0·3	—	—
Total calcium (mg.)	273	974	50	600	50	600	440	437
Total phosphorus (mg.)	680	696	347	346	484	469	630	554
Ca: P ratio	1:2·5	1:0·7	1:6·9	1:0·6	1:9·7	1:0·78	1:1·43	1:1·27

by Gilbert and Gillman[1] (G. & G. diet) and those of other diets which were essentially similar to the G. & G. diet, but modified in respect of certain mineral and vitamin contents.

MATERIALS AND METHODS

Experimental Diets

The compositions of the experimental diets (I - VIII) are shown in Table I. The general plan of the experiment was to compare the effects of the G. & G. diet (diet I) with those of (*a*) 5 other diets (diets II - VI) in which the Ca:P ratio was varied; (*b*) another diet (VII) which contained a more complete mineral mixture than the one used by Gibert and Gillman; and (*c*) a diet (VIII) in which the Steenbock 40 mineral mixture and food yeast moieties were replaced by the more complete mineral mixture included in diet VII and a synthetic vitamin mixture, respectively.

Diet I was equal in composition to the diet used by Gilbert and Gillman. It consisted mainly of dried maize meal porridge to which was added cooked-and-dried, hexane-extracted soya-meal as a source of extra protein; food yeast to supply additional quantities of B-complex-vitamins; cod-liver and wheat-germ oils as sources of the fat-soluble vitamins (A, D and E); and Steenbock 40 mineral mixture to furnish extra minerals. Diet II was essentially the same as diet I, but an additional quantity of $CaCO_3$ was added to raise the Ca:P ratio to the level of 1:0·8, the latter ratio being currently considered optimal for the rat.[11] In diets III - VI the standard mineral mixture was replaced with, firstly, NaCl (diet III); secondly, NaCl plus $CaCO_3$ (diet IV); thirdly, NaCl plus KH_2PO_4 (diet V); and fourthly, NaCl plus $CaCO_3$ plus KH_2PO_4 (diet VI). In the case of diet VII the composition was again very similar to that of the G. & G. diet (diet I), the only difference being that a more complete mineral mixture,[12] which contained more calcium, less phosphorus and a wider range of trace elements than the Steenbock 40 mixture, was used. The composition of diet VIII was essentially the same as that of diet VII, except that the food yeast moiety was replaced by the addition of a standard synthetic vitamin mixture.[13]

Animal Experiment

The experimental animals were 80 weanling albino rats bred at the NNRI and divided into 8 groups of 5 males and 5 females each. The rats were housed separately in individual screen-bottom cages in a spacious animal room in which the room temperature was kept at 27 ± 1°C and relative humidity at 50 ± 5%. Each group was fed one of the 8 experimental diets *ad libitum* for a period of 200 days, distilled drinking water being supplied regularly. Body-weights were recorded at regular intervals twice weekly.

At the end of the 200-day period the animals were killed with ether and their kidneys and femurs were dissected out, blood and other tissues being removed as thoroughly as possible. One kidney was preserved in buffered formalin for histological examination. The remaining kidney and both femurs were dried in an air circulation oven at 100°C for 24 hrs., the dried kidneys of each rat group being pooled into a single sample, for chemical analysis.

Histochemical and Chemical Methods

Kidney sections of 10 μ thickness were stained with alizarin for identification of calcium structures according to the method of Pearse.[14]

The calcium contents of kidneys and femurs were determined on dry-ashed specimens as oxalate, using a ceric sulphate and arsenic oxide titration method,[15] while magnesium was determined according to the EDTA-eriochrome black-T indicator method.[16]

The phosphorus content of wet-ashed specimens was determined according to the sodium molybdate-hydrazine sulphate technique.[17]

RESULTS

Relative Growth Rates of the Various Rat Groups

The growth rates of the rat groups fed on diets I - VI are shown graphically in Fig. 1. (The results obtained for groups VII and VIII were in all respects so similar to those obtained with the G. & G. diet—diet I—that it was considered unnecessary to include them in the figure.)

An outstanding feature of the growth is that, relative to the weight gains of the groups fed on the adequate- to high-calcium diets (I, II, IV and VI), weight gain dropped to a low level in the 2 groups which were fed on the low-calcium diets (III and V) from about the 35th day of the experiment.

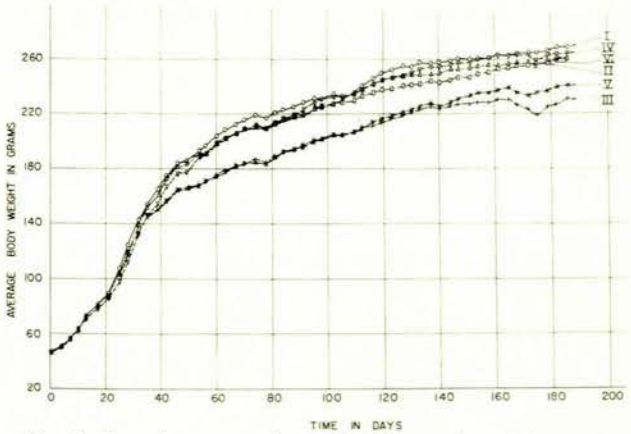

Fig. 1. Growth curves of rats fed the nephrocalcinogenic diet of Gilbert and Gillman[1] (diet I) and 5 other experimental diets (diets II - VI), showing growth depression in rat groups fed the low-calcium diets (diets III and V).

It is also noteworthy that high growth-rates, which are practically equal to that obtained with the G. & G. diet, could be obtained by feeding diets in which the more complete Steenbock 40 mineral mixture was replaced by only NaCl plus $CaCO_3$ (diet IV) or NaCl plus $CaCO_3$ plus KH_2PO_4 (diet VI).

Histochemical Observations

Calcium deposits in the kidneys of the type shown in Fig. 2 were found in the proximity of the cortico-medullary

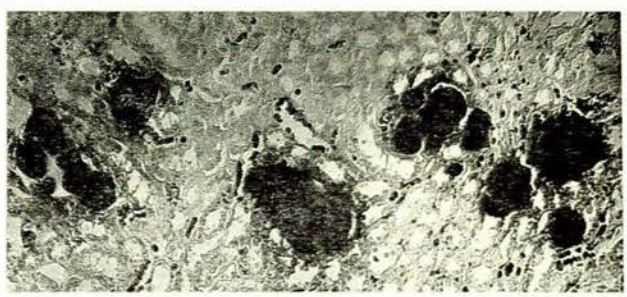

Fig. 2. Kidney calcification in rats fed on adequate-calcium, high-phosphorus diets.

junction of at least 7 out of 10 rats from each of groups I, VII and VIII. No signs of nephrocalcinosis were observed in any of the remaining 5 groups (II, III, IV, V and VI).

Mineral Composition of Kidneys and Femurs

In Tables II and III are shown the results obtained in the chemical analysis of kidneys and femurs, respectively,

of rat groups I - VI.

Scrutiny of these results shows that, in agreement with the histochemical findings, both the total ash and the calcium contents of the kidneys were appreciably higher in the case of the rats fed the G. & G. diet than those of the animals fed on the other diets. However, since the data were obtained on pooled specimens the statistical significance of the above differences could not be ascertained. In the case of the femurs, statistical analysis according to the non-parametric method of Wilcoxin[18] indicated significant differences in ash, calcium and magnesium content of the dried femur between group I (G. & G. diet) and those groups which received neither the full salt mixture nor additional $CaCO_3$ (diets III and V) (p, two-tailed, <1% in all comparisons). The calcium and magnesium contents of the ash fractions of the femurs appear not to have been influenced by the diet, since the small differences observed were insignificant (p, one-tailed, according to the Wilcoxin[18] test >6% in all comparisons).

TABLE II. MEAN ASH, CALCIUM, MAGNESIUM AND PHOSPHORUS CONTENTS OF KIDNEYS OF SEPARATE GROUPS OF 10 RATS EACH FED THE NEPHROCALCINOGENIC DIET (I) AND EXPERIMENTAL DIETS II—VI FOR A PERIOD OF 200 DAYS

	Composition of kidneys of rats fed diets I—VI.			
Experimental diet	*Ash/100 G dry kidney (G)*	*Calcium/ 100 G ash (G)*	*Magnesium/ 100 G ash (G)*	*Phosphorus/ 100 G ash (G)*
I	5·32	2·44	1·93	19·04
II	4·96	0·63	1·73	19·35
III	4·99	0·47	1·49	18·70
IV	4·90	0·49	1·70	19·55
V	4·90	0·55	1·77	17·98
VI	4·72	0·55	1·74	20·00

TABLE III. MEAN ASH, CALCIUM AND MAGNESIUM CONTENTS OF FEMURS OF SEPARATE GROUPS OF 10 RATS EACH FED THE NEPHROCALCINOGENIC DIET (I) AND EXPERIMENTAL DIETS II—VI FOR A PERIOD OF 200 DAYS

	Composition of femur of rats fed diets I—VI				
Experimental diet	*Ash/100 G dry femur (G)*	*Calcium/ 100 G dry femur (G)*	*Calcium/ 100 G ash (G)*	*Magnesium/ 100 G dry femur (G)*	*Magnesium/ 100 G ash (G)*
I	62·9	22·6	36·0	0·45	0·73
II	64·5	23·4	36·3	0·49	0·76
III	43·7	15·2	34·8	0·31	0·71
IV	62·8	22·7	36·1	0·40	0·63
V	44·6	15·3	34·3	0·37	0·82
VI	63·1	23·2	36·8	0·49	0·78

DISCUSSION

The results reported here confirm the finding of Gilbert and Gillman,[1] namely, that a diet consisting of maize to which is added cooked, defatted soya-meal, food yeast, Steenbock 40 mineral mixture and fat-soluble vitamins, causes nephrocalcinosis in growing rats. Furthermore, histochemical investigation of the kidneys of the experimental animals has shown that the nephrocalcinosis can be prevented by either increasing the calcium content of the G. & G. (diet II) or reducing it to a lower level (diets III and V). In the latter instances, however, it is clear from both the growth data and the ash and calcium contents of the femurs that reduction of the calcium content of the diet not only prevented the nephrocalcinosis, but also suppressed both growth rate and femur calcification (Fig. 1 and Table III).

Although no special attempts have been made to identify the mineral deposits in the kidneys, it can be seen from the data shown in Table II that the increase in kidney ash content is due mainly to an increase in the calcium content of the ash, indicating that calcium is undoubtedly the predominating inorganic component of these deposits.

The presence of mineral deposits in the kidneys of those rats fed modified G. & G. diets in which either the Steenbock 40 mineral mixture was replaced by a more complete mineral mixture (diet VII) or the food yeast by a standard vitamin B mixture, indicates that the nephrocalcinosis is not associated with a deficiency of trace elements or any specific property of food yeast.

At least one of the substances added to the maize meal porridge in this and Gilbert and Gillman's study must have nephrocalcinogenic properties, since no kidney calcification was observed by the latter authors when the diet consisted solely of dried maize porridge. Scrutiny of the recent literature on the subject of dietary nephrocalcinosis has revealed the importance currently attached to the role of the calcium : phosphorus ratio of the diet. Fourman[19] injected rats intraperitoneally on alternate days for 8 - 20 days with a sodium phosphate solution and within 8 days found calcium structures at the corticomedullary region in the kidneys. Other authors[5,20] have produced similar lesions by feeding diets which contained phosphorus in excess of the recommended optimal Ca : P ratio of about 1 : 0·8 or 600 : 500 mg./100 G diet.[21]

In agreement with these findings it was observed in the present investigation that all diets of which the calcium content complied with the NRC recommendation, or was even up to some 327 mg./100 G less, but of which the phosphorus content exceeded the recommended level (diets I, VII and VIII), caused kidney calcification. 'Correction' of the disproportionate Ca : P ratio, even where it entailed raising the calcium content to the very high level of 974 mg./100 G diet (diet II) eliminated the nephrocalcinogenic effect (diets II, IV and VI). It seems possible that by raising the calcium content to excessively high levels, as was done in studies on rats by Henneman[21] and Melick and Henneman,[22] the nephrocalcinogenic property might again be introduced. Moreover, the FAO/WHO[23] consider that excessive calcium intakes might interfere with the utilization of dietary iron, zinc and manganese. However, the high calcium level of diet II does not appear to have a detrimental effect on the growth rate and on either the histological structure of the kidney or the mineral composition of the femur.

The mechanism involved in the nephrocalcinogenic effect of adequate calcium-high phosphorus diets is by no means clear. O'Dell and co-workers[24] have found in guinea-pigs and rats, and Forbes[5] in rats, that the feeding of such diets produces femurs in which the magnesium content of the mineral component is lower than that of the femurs of control animals. In accordance with the finding of mineral deposits in the kidneys of magnesium-deficient animals by numerous workers,[5,20,25-28] Forbes[5] has suggested that the nephrocalcinosis caused by adequate calcium-high phos-

phorus diets is primarily due to a magnesium deficiency caused by an excessive phosphorus intake. However, a similar reduction in the magnesium content of femur ash was not observed in the present investigation (Table III).

Of particular interest is the finding that the nephrocalcinogenic effect was eliminated not only by 'correction' of the Ca:P ratio but also by reducing the calcium content of the diet to sub-optimal levels (diets III and V). This occurred even when the proportion of P relative to Ca was as high as 9·7:1 (diet V). It would therefore appear that although the nephrocalcinogenic effect might well be associated with the Ca:P ratio of the diet, a minimum level of calcium intake is required to produce mineral deposits in the kidney.

Although there is a difference in respect of calcium metabolism between rats and humans, it is, nevertheless, tempting to speculate on the basis of the results obtained in the feeding of calcium at low levels that the relatively low incidence of nephrocalcinosis among South African Bantu is the result of the very low calcium and relatively low phosphorus content of maize.[29] A recent radiological survey of hospital cases by Joffe[30] has revealed an exceptionally high incidence of both spinal osteoporosis and collapse of vertebral bodies, the author suggesting as one possible explanation the possibility of a long-continued negative calcium balance. It seems obvious that, should the hypothesis put forward above prove to be a valid one, the fortification of maize with calcium- and phosphorus-containing foodstuffs needs to be undertaken with the utmost care; an indiscriminate addition of small quantities of calcium and large quantities of phosphorus could well create a condition which is favourable to kidney calcification and thus cause widespread harm.

Whether or not the difference in calcium metabolism between rats and humans has a bearing on the problem of kidney calcification, is by no means clear. The difference may not necessarily be a difference in need and utilization at a cellular level, but merely in excretion, the faeces:urine calcium ratio being 2:1 in the case of humans and 22:1 in that of rats as was found by Wanner and co-workers.[31] However, should this difference in excretion be a justifiable objection to an extrapolation of the rat data to human nutrition, objections could be overcome by studying the nephrocalcinogenic effects of diets with the aid of monkeys. The latter species has been shown to excrete calcium in faeces and urine in a ratio equal to that of humans.[31]

SUMMARY

Experiments were carried out on growing rats in an attempt to identify those components of the nephrocalcinogenic diet of Gilbert and Gillman[1] (G. & G. diet) which produce the nephrocalcinogenic effect.

Eight groups of 10 growing rats each (5 males and 5 females) were fed separately on either the nephrocalcinogenic diet (diet I) or certain modifications of this diet (diets II-VIII) for a period of 200 days.

Conclusions are based on growth rates and the results of a histochemical investigation of the kidneys as well as of chemical determinations of the calcium and magnesium contents of kidneys and femurs.

Alizarin-positive deposits were found in at least 7 out of 10 rats in those groups fed on either the G. & G. diet or modifications of this diet, all of which contained (i) a certain minimum (at least 273 mg./100 G) of calcium and (ii) phosphorus in excess of the NRC-recommended ratio of Ca=1:P=0·8.

Ash and calcium contents were found to be higher in the kidneys which contained alizarin-positive material than in those which were free from such material, thus confirming the presence of mineral structures and indicating that calcium is the predominating inorganic component of the alizarin-positive material.

The nephrocalcinogenic effect of the G. & G. diet was eliminated by either correcting the Ca:P ratio through addition of calcium or reducing the calcium content of the diet to a level which was sufficiently low to suppress growth rate and optimal femur calcification.

The nephrocalcinogenic property of the G. & G. diet could not be eliminated through replacement of either the Steenbock 40 mineral mixture component by a more complete mineral mixture or the food yeast component by a standard vitamin mixture.

It appears that the nephrocalcinogenic property is associated with the Ca:P ratio of the diet, but that kidney calcification does not occur at sub-optimal levels of calcium intake, even when the diet is grossly imbalanced in respect of the Ca:P ratio.

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