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A LONG-TERM EXPERIMENT WITH RATS ON A HUMAN DIETARY

II. CALCIUM AND PHOSPHORUS DEPLETION AND REPLACEMENT

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(With 1 Figure in the Text)

It is with deep regret that we announce the death on February 8th of Professor C. C. Okell, who had edited this *Journal* since the retirement of the late Professor Nuttall at the end of 1937. A full Obituary will appear in our next number.

The experiments reported in the present paper were designed to determine to what extent the improvement in the dietary resulting from these supplements was due to their Ca and P contents. The original experiment of Orr *et al.* has been repeated under very similar conditions and with the same dietary. In their experiment two diets were used: the basal dietary and one consisting of this dietary with milk and green food added. In the present paper the experiment has been extended to include a third diet consisting of the basal diet with Ca and P added as salts in amounts equal to that present in the milk and green food. A preliminary account of the observations on animals bred from stock has already been published (Gaunt *et al.* 1938). It was felt, however, that further observations would yield results of greater value; and in the present paper the results obtained in three generations of rats are reported.

EXPERIMENTAL

The diets

The basal dietary (diet I) used in this experiment was in all respects the same as that employed by Orr *et al.* The individual constituents of the diet

were prepared in a manner similar to that adopted by humans, and different rations were given to the rats on each day of the week. In the original experiment it was found necessary to add double the amount of milk in the diet in order to eliminate the high mortality rate in lactating mothers. In the present experiment this increase in the milk allowance was not necessary and the diet was used without this addition.

In the original experiment the milk and green food supplements were not incorporated in the daily ration, but were given separately *ad lib.* It was noted, however, that, on the average, the rats consumed about 3·2 times the amount of milk present in the human dietary and 28 g. of fresh green food, kale, thousand-headed kale or outer cabbage leaves per 100 g. of solid foodstuffs. Since more strict comparisons between the rats fed on the different diets were desired in the present experiment, the necessary amounts of green food and milk were incorporated in the diet to give as homogeneous a mixture as possible.

Analyses for Ca and P were made daily on diets I and II over a period of a fortnight; the results are shown in Table I. No analyses were made on the

Table I. *Analyses of diets I and II on two successive weeks. Values per* 100 *g. dry weight*

		Diet I			Diet II		
Day		Ca	P	Ca/P	Ca	P	Ca/P
Monday	1	0·097	0·225	0·432	0·247	0·290	0·854
	2	0·135	0·227	0·593	0·367	0·278	1·319
Tuesday	1	0·112	0·276	0·408	0·290	0·357	0·812
	2	0·120	0·315	0·381	0·238	0·378	0·630
Wednesday	1	0·126	0·260	0·487	0·235	0·327	0·718
	2	0·108	0·211	0·513	0·235	0·266	0·886
Thursday	1	0·083	0·136	0·612	0·250	0·262	0·954
	2	0·091	0·150	0·605	0·346	0·262	1·318
Friday	1	0·113	0·323	0·348	0·241	0·567	0·426
	2	0·130	0·343	0·377	0·280	0·399	0·702
Saturday	1	0·165	0·246	0·669	0·295	0·295	1·000
	2	0·170	0·278	0·611	0·378	0·358	1·055
Average		0·121	0·249	0·503	0·284	0·338	0·890

Sunday's ration which in each group consisted solely of bread and water. Each daily ration, apart from that of Sunday, contained at least seven different constituents, the composition of which would unavoidably alter from time to time. In addition sampling of the mixtures was not easy. It is felt, therefore, that the agreement between the analyses of similar rations prepared on different days is quite good.

It will be seen that the Ca and P contents of diet I are very low, and, although the addition of the green food and milk raised the average Ca content from 0·121 to 0·284% and the P content from 0·249 to 0·338%, the supplemented diet is still low in these elements. The Ca/P ratio of diet II is, however, much the more favourable for normal calcification.

Diet III was constructed by adding to diet I enough Ca lactate and Na_2HPO_4 to raise the average Ca and P contents to those of diet II. It was

impracticable to add different amounts of Ca and P salts on each day owing to the variations that existed in each diet on a given day from week to week. The Ca and P contents of diet III were therefore adjusted by taking the average difference between twelve samples of diets I and II. This necessitated the addition to diet I of 0·324 g. of Ca lactate (B.P.) and 0·105 g. of Na_2HPO_4 (analar) per 100 g. wet weight.

The diets and tap water were given *ad lib.*

The diets were analysed directly only for Ca and P, but the complete composition was calculated from the tables giving the calorie, protein, fat, carbohydrate, mineral and vitamin contents of foods used by the Carnegie Trust Dietary Survey (*Tech. Commun. Bur. Anim. Nutrit., Aberd.*, no. 10, 1938; Fixsen & Roscoe, 1938). The results of this analysis are given in Table II.

Table II. *Calculated composition of diets per* 100 *g. dry weight*

Diet	Calories	Protein	Carbo-hydrate	Fat	Ca	P	Vitamins I.U. A	B_1	C	D
I	466	13·6	77·2	10·0	0·092	0·226	156	80	116	14
II	472	16·5	71·9	11·7	0·261	0·305	1380	122	517	15

These figures do not differ materially from those calculated by the previous authors (Orr *et al.* 1936); it is probable that they approximate fairly closely to the true values since the Ca and P contents correspond quite well with those found by chemical analysis. Diets I and II contained 74 and 78% of water respectively.

It was virtually impossible to give any figure for the daily vitamin intake, but some investigations were undertaken to see if this was adequate in the case of A and B_1. Irving & Richards (1938) have shown that degeneration of the funiculus praedorsalis occurs in rats in vitamin A deficiency. The medullas of several of the rats on diet I were examined but no evidence of degeneration was found. The vitamin A content would thus appear adequate. According to Drummond (1938), 4–5 I.U. of B_1 per day are adequate for satisfactory growth in rats. From the figures given in Table II it might appear that the vitamin B_1 content of diet I was adequate were it not for the fact, determined by one of the authors (W. T.), that a daily supplement of 0·3 I.U. to the diet definitely increased the growth rate. This discrepancy remains at present unexplained. Diet II would in all probability have an adequate B_1 content. The clinical condition of the rats did not indicate any marked deficiency in other constituents of the B complex.

Vitamin C is of questionable value for rats.

The requirement of vitamin D is so intimately bound up with the levels of intake of Ca and P and the Ca/P ratio that it is impossible to state any figure for adequacy in rats. In diet I the levels of intake of Ca and P were low and the Ca/P ratio rachitogenic; complete calcification would probably not have occurred whatever the intake of vitamin D.

Rats

Lister Institute hooded rats of the same strain as those used in the original experiment were employed. The first generation was bred from stock. Three groups were used, group I being given diet I, etc. In the first generation, forty-eight rats per group were employed. Of these, twelve males and twelve females were kept for breeding and four males and four females were killed for examination at 40, at 70 and at 100 days of age. In the second and third generations each group consisted at least of fifty-six rats. Of these, four males and four females were examined at weaning (21 days), at 40, at 70 and at 100 days of age and the rest were kept for breeding. The third generation was mated to obtain data of their reproductive capacity and the experiment ended with the weaning of the fourth generation.

Owing to the fact that parents of the first generation were all on stock diet, it was possible in almost all cases in the parental generation for a male rat in group I to have a female litter-mate in the same group; and, in addition, for these two rats to have a male and a female litter-mate in both groups II and III. It was thus possible to compare litter-mates of the same age and both sexes in different groups. This was of course impossible with the second and third generations.

Other details

The rats were housed, not in large cages as in the original experiment, but in cages holding four rats each; all three groups were kept in the same room at a temperature of 68–70° F. The litters were weighed in bulk about 6 hr. after birth and individually at weaning (21 days); the 1st generation was weighed twice weekly thereafter and the second and third generations weekly. The animals for examination were killed with coal gas and radiographed. The upper and lower incisor teeth and the bones of the right leg were then dissected out. The upper incisors were used for histological examination and the lower incisors and femur, tibia and fibula were reserved for chemical analysis.

Results

Growth rate

The average growth rates for males and females up to 119 days of age are shown in Fig. 1. These curves were obtained from the average weight of those rats which lived for 119 days or more. Rats which were killed for examination at the times stated or for other purposes, or rats which died from unknown causes, were rejected from the averages. Fluctuations which might have been brought about by changes in the environmental conditions were to a large extent ruled out by the fact that the rats were not all of the same age on the same day. For comparison the growth rate of Rowett Institute stock rats is superimposed; these rats were similarly housed and were fed on an optimal diet (Thomson, 1936).

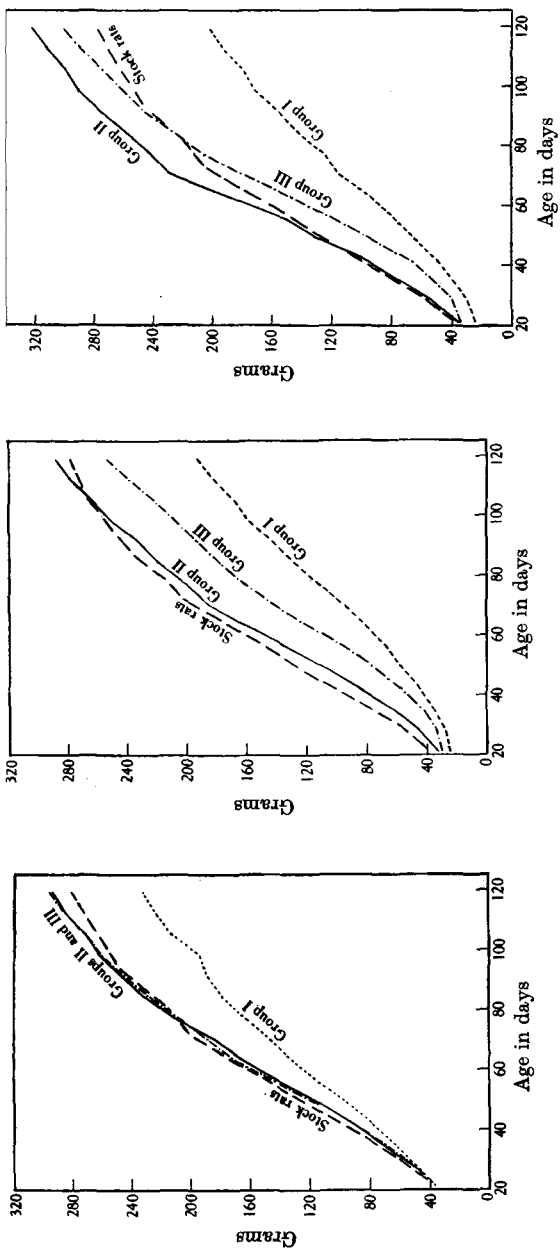

Fig. 1. Average growth curves of male and female rats in all generations. The average growth curve of stock rats has been superimposed in all cases.

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In the first generation males of both groups II and III grew at identical rates, this rate being approximately the same as that of contemporary stock rats.

In the second generation the group II males grew rather more slowly than stock rats but reached their weight after 110 days. The group III animals, however, grew at first quite slowly, being nearly 50 g. below the weight of the stock rats at 70 days. After this age they grew at a more normal rate but they were always about 40 g. lighter than the corresponding stock rats.

In the third generation the growth rates of both groups II and III males were markedly better than in either of the preceding generations. For about 50 days the group II animals grew as rapidly as stock rats; after that they grew even more rapidly so that at 70 days they were nearly 20 g. heavier, an advantage which was maintained up to and beyond 119 days. The group III rats did not at first grow as well as stock rats; but after 50 days the growth rate increased until at 90 days they were as heavy as stock rats and after that exceeded them in weight.

Comparison of the growth curves of the female rats in groups II and III with the female stock rats showed differences similar to those obtained with the male rats in these groups. In the first generation the group II and III rats grew at almost identical rates, approximately the same as female stock rats. In the second generation the group II rats grew at the same rate as stock rats while the group III animals were always lighter than stock. As in the case of the males, in the third generation group II females exceeded the weight of stock rats after 45 days and were about 20 g. heavier than stock rats from 80 days onwards. On the other hand, the group III animals grew at first quite slowly; their rate of growth increased, however, until they were as heavy as stock rats at 75 days and as group II animals at 119 days.

In all three generations group I rats grew very badly. Their growth was best in the first generation where a weight of 232 g. was attained by the males in 119 days in comparison with 293 and 294 g. in groups II and III respectively. In the second and third generations a weight of 200 g. was barely attained at 119 days; the lowest weight of group III animals at this age was 254 g. The third generation animals grew at a slightly higher rate than did those in the second generation. The group I females in each generation showed changes qualitatively the same.

These results showed that in rats bred from stock, the increased growth rate obtained by supplementing the poor diet with milk and green food could equally well be got with supplements of equivalent amounts of Ca and P as salts. In further generations this equality was not maintained though in the third generation the growth rate of group III did not fall far short of that of group II.

General appearance

The rats in group I appeared much as in the original experiment (Orr *et al.* 1936). They had a stunted appearance with staring coats and many of them showed marked depigmentation of the fur. The rats in groups II and III were

nearly indistinguishable to the eye; depigmentation occurred to a certain extent in group II and to a lesser degree in group III but by 100 days both groups had normal dark sleek coats. Many of the group I rats had incisor teeth with a china-like opacity and little or no orange pigment; these teeth were brittle and malocclusion was common in the older rats. In no case did the incisor teeth of group II or III rats appear abnormal to the naked eye; they were translucent and had a deep orange pigment.

Appetite

No check on the food consumption was kept, but there was no doubt that this was much higher in groups II and III than it was in group I. This effect of Ca and P in stimulating the appetite has also been noted by Rottensten (1938).

Death-rate

The death-rate in all groups after weaning was negligible. The high mortality in group I rats reported in the original paper was due to an intercurrent Salmonella infection which had ceased before this experiment was begun.

X-ray photographs

In the case of the first generation, when litter-mates could be compared, striking differences were found in the degree of calcification of the skeleton which were easily detected in the X-ray films, group I being poorly calcified in comparison with groups II and III in which the density of the shadows of the bones appeared indistinguishable.

In order to detect whether rickets was present, the upper end of the tibia was compared with the radiographic scale of Bourdillon *et al.* (1931). This reads from 0, or extreme rickets to 12, or complete healing. The epiphysis of the upper end of the tibia does not close till 37 months of age (Donaldson, 1924), but comparison with this scale was not found possible after 100 days of age. The metaphyses of group I animals were wider than those of groups II and III and the upper end of the diaphysis showed some degree of blurring but in no case was marked rickets present. The lowest reading obtained in group I was 8 on the scale but most of them lay between this figure and 11, the lower reading being got with the younger rats. The degrees of calcification in groups II and III were identical and higher, lying between 10 and 12. None of the rats in any group showed epiphyseal swelling.

Chemical analysis

The right femur, tibia and fibula and the lower incisors were removed immediately after the rat had been X-rayed; the bones and teeth were scraped as far as possible free from adherent soft tissue and the larger bones were split. These were extracted with hot alcohol for about 16 hr. and with ether for a further 12–16 hr. The material was then dried at 100°. The bones were heated in an electric muffle at dull red heat until ashing was complete. The ashing of

the teeth was facilitated by the addition to partially ashed material of a few drops of conc. HNO_3. All the nitric acid was removed before the ashing of the nitrated tooth was completed in the muffle. Silica crucibles were used. In every case the ash was weighed 2 hr. after it had been removed from the furnace and placed over anhydrous $CaCl_2$. The bone and tooth ash was then dissolved in a few ml. of hot approx. 5*N* HCl, the ash solution being diluted to a known volume containing approximately 1 mg. of ash per ml. of solution. Calcium and phosphorus determinations were made in duplicate on aliquots of the ash solutions. Since only slight and inconsistent differences in the percentage content of Ca and P in the tooth and bone ash could be observed between corresponding rats in the different groups and generations, only figures for the ash contents of the bones and teeth are quoted.

In Table III are given the average percentage ash contents of the dry fat-free bones and teeth of the rats examined; the corresponding ash values of bones and teeth of stock animals of comparable ages and on normal diets are included. The absolute weights of the bone and tooth ash are also given and are expressed as percentages of the stock rat figures. The last value is not obtained from the average ash weights of the four rats examined in each instance but from curves expressing the relationship between body weight and bone ash and tooth ash weights for the whole group. By this means it is possible to obtain a truer comparison since in several cases the average weights of the four rats chosen for analysis differed from the average weight of the rats of that group at that particular age.

Bones

The table shows that in none of the groups were the bones of the experimental animals as well calcified as those of the contemporary stock rats. Stock rats of both sexes had a bone ash of about 50% at weaning, and of 65% at 100 days; at 70 days they had bones better calcified than those of any group of experimental animals at 100 days. Thus, though the rats on diet II grew at a rate equal to or greater than that of stock rats, they did not lay down inorganic bone substance at a correspondingly rapid rate and calcification was much delayed. This is shown even more clearly by a comparison of the absolute ash values.

The ash percentages of the bones of group II and III animals showed quite a remarkable correspondence. In the first generation in both sexes the bones of those groups of rats were equally as well calcified; even in the second and third generations, when the group III animals did not grow at the same rate as the group II rats, the differences in the ash percentage of the bones were very slight. The difference in growth rate in the second and third generations is reflected in the differences in the absolute bone ash values.

On the other hand, the ash percentages of the bones of the group I rats was always markedly less than in the corresponding group II and III animals and much less than in stock rats. Calcification in group I rats was best in the

Table III. *Chemical analysis of bones and teeth*

			1st generation			2nd generation			3rd generation			1st generation			2nd generation			3rd generation		
Age	Sex	Group	Ash % bone	Wt. ash	Ash expressed as % stock	Ash % bone	Wt. ash	Ash expressed as % stock	Ash % bone	Wt. ash	Ash expressed as % stock	Ash % tooth	Wt. ash	Ash expressed as % stock	Ash % tooth	Wt. ash	Ash expressed as % stock	Ash % tooth	Wt. ash	Ash expressed as % stock
21	M.	1	—	—	—	37·20	0·015	58	33·44	0·014	54	—	—	—	73·94	0·017	—	72·80	0·016	—
		2	—	—	—	41·75	0·030	115	38·01	0·033	127	—	—	—	76·62	0·022	—	72·30	0·021	—
		3	—	—	—	41·92	0·025	96	35·52	0·028	108	—	—	—	74·84	0·022	—	72·63	0·022	—
		Stock	—	—	—	48·51	0·026	100	48·51	0·026	100	—	—	—	—	—	—	—	—	—
	F.	1	—	—	—	36·61	0·015	56	35·59	0·016	59	—	—	—	73·63	0·018	—	72·65	0·017	—
		2	—	—	—	40·98	0·027	100	39·51	0·030	111	—	—	—	74·84	0·020	—	72·43	0·020	—
		3	—	—	—	41·92	0·027	100	39·69	0·027	100	—	—	—	73·56	0·021	—	70·36	0·021	—
		Stock	—	—	—	47·06	0·027	100	47·06	0·027	100	—	—	—	—	—	—	—	—	—
40	M.	1	36·06	0·048	38	37·90	0·022	18	33·44	0·024	19	70·88	0·037	82	73·38	0·026	58	74·81	0·027	60
		2	42·94	0·092	74	48·37	0·080	64	49·82	0·105	84	72·70	0·042	93	77·57	0·038	82	76·90	0·046	102
		3	43·60	0·086	69	46·77	0·050	40	43·80	0·051	41	73·09	0·048	107	77·83	0·034	76	76·57	0·038	84
		Stock	54·75	0·125	100	54·75	0·125	100	54·75	0·125	100	75·60	0·045	100	75·60	0·045	100	75·60	0·045	100
	F.	1	38·58	0·048	40	37·98	0·025	21	35·94	0·026	21	72·63	0·034	68	73·81	0·024	48	75·03	0·025	50
		2	44·79	0·080	66	48·41	0·075	62	50·20	0·090	74	73·64	0·037	74	76·25	0·035	70	75·05	0·039	78
		3	43·82	0·077	64	45·65	0·046	38	47·58	0·058	49	74·01	0·042	82	75·86	0·033	66	76·56	0·038	76
		Stock	54·91	0·121	100	54·91	0·121	100	54·91	0·121	100	75·47	0·050	100	75·47	0·050	100	75·47	0·050	100
70	M.	1	44·88	0·117	36	36·51	0·060	18	37·08	0·068	21	77·16	0·061	71	73·37	0·042	49	71·16	0·045	52
		2	55·56	0·237	72	57·12	0·235	72	58·28	0·298	91	76·94	0·083	96	79·21	0·072	84	78·98	0·092	107
		3	56·10	0·255	78	53·24	0·162	50	55·38	0·220	67	78·40	0·082	95	77·20	0·066	77	77·24	0·077	90
		Stock	62·77	0·327	100	62·77	0·327	100	62·77	0·327	100	77·69	0·086	100	77·69	0·086	100	77·69	0·086	100
	F.	1	46·50	0·100	36	39·03	0·058	21	40·64	0·075	27	77·53	0·053	62	71·98	0·039	46	70·12	0·045	53
		2	56·03	0·194	70	58·00	0·200	72	58·06	0·230	83	79·50	0·072	85	77·45	0·072	85	78·24	0·082	96
		3	58·42	0·202	73	56·68	0·150	53	58·50	0·200	72	78·98	0·073	86	77·72	0·062	73	77·67	0·072	85
		Stock	64·65	0·276	100	64·65	0·276	100	64·65	0·276	100	77·31	0·085	100	77·31	0·085	100	77·31	0·085	100
100	M.	1	51·78	0·177	40	44·75	0·123	28	51·03	0·138	31	75·44	0·078	70	74·68	0·062	56	76·27	0·066	60
		2	60·41	0·391	88	62·22	0·370	83	64·10	0·480	108	77·93	0·115	104	79·18	0·110	99	79·11	0·127	114
		3	62·70	0·417	94	62·19	0·300	67	63·60	0·410	92	78·20	0·109	98	79·28	0·088	79	78·73	0·107	96
		Stock	64·84	0·445	100	64·84	0·445	100	64·84	0·445	100	78·49	0·111	100	78·49	0·111	100	78·49	0·111	100
	F.	1	52·06	0·142	40	46·34	0·104	29	47·56	0·120	34	75·77	0·068	68	71·54	0·055	55	73·28	0·060	60
		2	61·88	0·296	83	63·60	0·275	77	63·90	0·335	94	78·63	0·098	98	79·45	0·092	92	78·72	0·101	101
		3	62·75	0·305	86	62·57	0·232	65	63·40	0·306	86	78·65	0·095	95	79·28	0·079	79	79·26	0·095	95
		Stock	65·25	0·356	100	65·25	0·356	100	65·25	0·356	100	77·41	0·100	100	77·41	0·100	100	77·41	0·100	100

first generation. Only in the 100-day rats of the first generation and the 100-day males of the third generation did the ash content of the bones of group I exceed 50%. The absolute ash values showed even more clearly how poorly the group I rats grew and calcified. In the first generation the ash value did not exceed 40% of that of stock rats and in subsequent generations it was only 18–34%.

Teeth

The percentage ash in the teeth did not show such striking differences as did the bone ash; in the first generation in particular there was little variation from the stock rat figures, even in group I animals. In the second and third generations, however, except for the animals taken at weaning, more marked differences were obtained between the group I rats and those of groups II and III and stock; the last three were always indistinguishable from each other. In both these generations the percentage ash content of the teeth of group I animals was higher at 40 days than at 70 days. This is probably due to the fact that as the rat incisor replaces itself in about 40 days, the teeth at 40 days of age contained over 50% of material of normal ash content laid down prior to weaning, whereas the 70-day animals had teeth composed entirely of material calcified whilst the animals were eating the poor diet.

Comparison of the absolute weights of the tooth ash show that in every case the experimental rats had teeth more nearly equal to the stock rat value. In general, group II rats had a tooth ash slightly heavier than that of group III animals and almost as heavy as that of stock rats. Group I rats, apart from the weaning animals, which had teeth of normal weight and inorganic content, always had lower tooth ash values than the corresponding stock animals. This difference is more apparent in the second and third generations than in the first. However, where in the case of the bones the ash value was only about 20% of the stock rat value, the tooth ash was about 50%. From these figures and to a lesser extent from those of groups II and III it seems natural to conclude that Ca and P are more readily available for tooth formation than for ossification.

The results obtained from chemical examination of the bones and teeth of groups II and III show that Ca and P in inorganic form can induce calcification as effectively as that present in milk and green food.

Histological examination of the teeth

The frequent abnormality of the group I rats' teeth noted above seemed to call for closer enquiry and the incisor teeth of all the rats killed were examined microscopically. Schour & Ham (1934) and Rosebury & Karshan (1931) and many others have pointed out that the constantly growing incisor tooth of the rat offers a very delicate index of calcification. Under normal conditions the daily increment of dentin is laid down as a narrow strip 16–20μ wide and the dentin formed from this is regular in structure. When calcification is impaired, the predentin is abnormally wide, the juncture with dentin is tortuous in

outline, interglobular spaces are seen in the dentin and in extreme cases vascular inclusions occur in the predentin and dentin.

In the present paper, the upper incisor teeth were decalcified and embedded in paraffin; longitudinal sections were cut and stained with haematoxylin and eosin. The predentin width at the apical end of the tooth was measured with a micrometer eyepiece and any abnormality was noted.

The average predentin widths for males and females over the three generations are given in Table IV. It will be noted that at weaning the widths were normal in all groups; and in groups II and III, with a few exceptions, the widths were normal over the whole experiment. This confirms the chemical findings and shows that Ca and P in inorganic form can also induce the formation of histologically normal teeth.

Table IV. *Predentin width* (μ) *of the upper incisor teeth*

Age ...		Weaning			40 days			70 days			100 days		
Generation ...		1	2	3	1	2	3	1	2	3	1	2	3
Group I	M.	—	18	20	29	27	34	41	35	41	38	23	34
	F.	—	18	20	31	33	34	34	32	45	25	39	43
Group II	M.	—	18	20	25	27	20	23	17	18	20	21	21
	F.	—	18	20	20	23	19	22	18	17	20	21	21
Group III	M.	—	18	20	22	22	23	19	21	19	21	20	22
	F.	—	18	19	21	20	27	19	18	19	21	23	20

In group I the predentin width rose with increasing age after weaning, indicating progressive malcalcification. This was probably most extreme at 70 days. A comparison of these results with the values obtained for tooth ash in group I show that the chemical changes, though usually of the same order, are in general much slighter. Karshan & Rosebury (1933) and others have pointed out that the histological appearance of teeth is a more sensitive index of dietary influences than chemical analysis, the calcification process being retarded at this level of intake but not ultimately interfered with to any marked extent. Only with diets of a much lower Ca content than those used in this experiment does the ash content of the tooth fall markedly (Templin & Steenbock, 1933).

The occurrence of vascular inclusions forms a good check on the accuracy of the predentin measurement. None of the group II or III rats showed vascular inclusions; the group I rats showed none at weaning in any generation, but thereafter the number rose steadily with age in all generations, the 70-day teeth having the highest incidence.

Reproductive performance

Rats were mated at about 119 days of age and complete breeding data were kept of each group. In all cases, litters of over eight rats were reduced to that number immediately after birth. A summary of the results is given in Table V.

Table V. *Summary of reproductive performance*

Generation	Group	No. mated	No. of litters found	Days from introduction of male to birth of litter	Av. wt. at birth g.	Av. born per litter	Mortality at birth %	No. removed at birth	Av. weaning weight (g.)		Av. weaned per litter	Mortality birth to weaning %	No. of young weaned per doe mated
									Males	Females			
1st	1	12	10	48	5·30	8·7	?	15	24·8	23·8	7·0	2·8	5·9
	2	12	12	34	5·36	9·7	0·9	21	32·0	32·0	7·5	4·2	7·5
	3	11	11	26	5·48	10·0	0·9	20	29·6	28·4	7·9	2·2	7·9
2nd	1	19	18	31	5·23	6·1	6·4	7	23·7	23·5	7·1	11·5	4·5
	2	12	12	29	5·40	9·3	Nil	23	35·3	34·0	7·0	5·6	7·0
	3	11	11	29	5·56	9·3	0·9	19	32·0	29·3	7·1	6·0	6·5
3rd	1	12	9	43	5·37	6·1	5·4	1	26·3	27·6	4·9	33·3	2·8
	2	12	12	32	5·41	9·8	1·7	23	34·1	34·3	7·6	9·6	7·0
	3	12	12	34	5·30	9·5	3·5	18	29·6	27·3	7·3	5·4	7·3

It will be seen that almost the only value in which group I rats equal groups II and III is that of the average birth weights. The group I rats mated slowly and had smaller litters, as can be seen both from the average born per litter and also the number that had to be removed to reduce litters to eight. The small litters are probably responsible for the good weaning weight. In the second generation, nineteen females were mated in group I as it was feared that there would not be enough young for the third generation. The weaning weights were low save in the third generation but here the litters were abnormally small at weaning owing to the high mortality rate from birth to weaning.

The reproductive performance of groups II and III was much better than that of group I but was by no means optimal. The average born per litter was good; the weaning weights were also good but lower in group III; the mortality rate, however, both at birth and from birth to weaning was high. It should be stated here that the birth mortality figures should be accepted with caution in all groups as no check was possible on mothers who ate their young before they could be seen, a by no means rare occurrence. The figures for the mortality from birth to weaning are much more reliable and show that even in groups II and III lactation was not completely normal.

The best insight into the reproductive performance of all groups is got from the last column, that of the number of young weaned per doe mated; the figures for groups II and III remain constant from generation to generation but those of group I get progressively worse.

The group III animals of the first generation appeared to undergo pregnancy and lactation so satisfactorily that it was decided to kill and analyse the mothers in the second and third generations after the young had been weaned, using non-pregnant animals of the same group as controls. The average age of the animals used was 170 days. The bones and lower incisor teeth were analysed for ash and the predentin width was measured in the upper incisor teeth. The chemical findings are shown in Table VI, the ash values of 100-day rats in the different groups being included for comparison.

A perusal of the ash figures reveals the interesting fact that pregnancy and lactation had interfered markedly with bone calcification in all groups, most so in group I. The absolute bone ash values in all groups, save in group I, third generation, showed no advance on those obtaining at 100 days though the rats were on an average 70 days older, and were much lower than those of control rats. In every case, however, the absolute tooth ash figures had risen and were much nearer those of the control animals. It would thus appear that pregnancy and lactation interfere much more with bone than with tooth calcification and, moreover, that tooth calcification can occur at the expense of bone. This seems so significant a finding, taken in conjunction with that of a previous section, that it is intended to investigate further the relative calcification rates of teeth and bones.

The predentin width figures (Table VII), like those reported in a previous section, are much more revealing of tooth calcification than are the ash figures.

Table VI. *Bone and tooth ash figures of lactating rats and controls*

	No. rats		Wt. bone	Wt. ash	Ash % bone	Wt. tooth	Wt. ash	Ash % tooth	Av. no. of rats weaned per litter
				2nd generation					
Group 1	4	100 day	0·2028	0·0939	46·34	0·0699	0·0479	71·54	8
	4	Lactating	0·1929	0·0961	49·79	0·1095	0·0840	76·71	
	3	Virgin	0·3160	0·1931	60·09	0·1335	0·1050	78·66	
Group 2	4	100 day	0·4709	0·2997	63·60	0·1338	0·1062	79·45	8
	4	Lactating	0·4519	0·2879	63·71	0·1596	0·1262	79·11	
	2	Virgin	0·5823	0·3912	67·19	0·1783	0·1418	79·53	
Group 3	4	100 day	0·4104	0·2570	62·57	0·1112	0·0881	79·28	8
	3	Lactating	0·4166	0·2556	61·08	0·1575	0·1252	80·12	
	2	Virgin	0·5573	0·3707	66·54	0·1727	0·1370	79·35	
				3rd generation					
Group 1	4	100 day	0·1956	0·0966	47·56	0·0588	0·0430	73·28	5
	4	Lactating	0·2665	0·1532	57·26	0·1285	0·1002	78·49	
	4	Virgin	0·4386	0·2725	61·92	0·1399	0·1120	79·97	
Group 2	4	100 day	0·5001	0·3196	63·90	0·1244	0·0979	78·72	8
	4	Lactating	0·4621	0·2943	63·77	0·1572	0·1246	79·28	
	4	Virgin	0·6382	0·4355	68·29	0·1688	0·1348	79·87	
Group 3	4	100 day	0·4415	0·2801	63·40	0·1121	0·0888	79·26	7
	4	Lactating	0·4298	0·2721	63·30	0·1454	0·1153	79·36	
	—	Virgin	—	—	—	—	—	—	

Table VII. *Predentin width* (μ) *in the upper incisor teeth of lactating and virgin rats*

Generation ...	2nd		3rd	
Group	Lactating	Virgin	Lactating	Virgin
I	52	19	27	21
II	27	26	28	22
III	30	23	27	—

Tooth calcification was impaired in the pregnant animals in both generations, compared with the controls. In the case of groups II and III, the difference was not, however, marked. In group I second generation, the predentin width was extreme, being the highest figure obtained in the whole experiment. In the third generation, however, the value of the pregnant group I animals was no worse than with groups II and III. A glance at Table VI shows that there is a considerable difference in the bone ash figures of the group I pregnant animals in the second and third generation, that of the latter being much higher; this is probably to be explained by the fewer young weaned by the third generation rats.

Discussion

The value of the data presented in this paper can only be assessed after comparison is made with the results obtained in other nutritional experiments.

The Ca content of diet I, 0·121%, is apparently sufficient to allow rats to grow and reproduce, although their performance is subnormal. According to Campbell *et al.* (1935) only one generation of rats can reproduce on a diet

containing 0·1% Ca, the second generation being sterile. On the other hand, a level of 0·19% permitted growth and reproduction to take place over several generations, although at subnormal rates: increase in the Ca content of the diet improved its nutrition quality still further (Sherman & Campbell, 1935). Thus it appears that the Ca content of diet I is just at the threshold level for reproduction through several generations and with a poorer diet the present experiment would not have been possible.

Shohl & Wolbach (1936) found that the effects of the ratio of Ca to P were intimately related to the level of intakes of Ca and P. They produced a graph expressing this relationship from which the rachitogenic value of a given diet could be computed. Reference to this diagram shows that diet I is moderately rachitogenic and this is confirmed by the X-ray findings reported above. If, however, the A/R ratio (ratio of bone ash to organic matter) is calculated, it is found that many of the group I rats should show marked rickets. Chick *et al.* (1926) stated that an A/R ratio lower than 0·5 denoted severe rickets, 0·5–0·7 moderate rickets, and only at levels above 1·0 was rickets absent. Several of the group I rats had rachitic A/R ratios but none of them showed marked rickets. Since, however, many of the group I rats had generalized osteoporosis it would appear that chemical findings alone cannot distinguish between this condition and frank rickets.

The Ca and P levels of diets II and III were quite adequate for growth and reproduction: Sherman & Campbell (1930) found that a diet containing 0·2% Ca sufficed to maintain a rat colony over twenty-one generations. The curve of Shohl & Wolbach places both these diets well outside the rachitogenic range.

The findings with the group I rats were similar to those of the original paper, except for the absence of any high mortality after weaning. These rats appeared in poor condition to the eye; they had a low growth rate and a poor reproductive capacity; they had a slight incidence of rickets, were poorly calcified and had defective tooth formation.

The performances of group II and III rats were virtually identical save for differences in growth rate in the second and third generations; their degree of calcification and tooth formation were the same, their reproductive performances did not differ markedly, and they were indistinguishable to the eye. It is of interest, however, to note that the bone ash values fell well below those of stock animals of the same age, so that in neither case were the diets optimal; the X-ray findings confirmed that calcification was not always perfect. This finding for group II rats, which grew at least as well as stock rats in all generations, corresponds with that of Sherman & Booher (1931) who fed growing rats on diets containing varying amounts of Ca from 0·16 to 0·50%. The animals all put on weight at much the same rate but differed markedly in their total Ca content. As they became adult their Ca contents approached the same figure. The same happened with the rats in the present experiment since the ash values of non-pregnant control animals at 170 days in group I

were far higher than at 100 days and in groups II and III approached the normal figure for adult rats, about 68%.

The routine histological examination of the incisor teeth, a new departure in nutritional studies, gave interesting information on the progress of calcification in organs not usually investigated. The results followed in general those found for chemical analysis. It seemed particularly interesting to note that the incisor teeth of all three groups appeared histologically identical at weaning in both the second and third generations, showing that the poor diet of the mothers in group I did not interfere with tooth formation in the young; only after the rats had to fend for themselves did the group I teeth suffer. On the other hand, the addition of either milk and green food or of Ca and P salts sufficed to ensure proper dental calcification up to adulthood. Since the calcification process in teeth in man is the same as that of the rat's incisor, this finding emphasizes strongly the importance of adequate intakes of Ca and P while the permanent dentition is being laid down.

The most significant finding reported above lies in the comparison of diets II and III, in the comparison of the nutritional values of milk and green food as against equivalent amounts of Ca and P salts.

It would be expected that the addition of Ca salts to a poor diet such as diet I would increase its nutritional value. Osborne & Mendel (1918) many years ago showed this to be true; Elliot *et al.* (1922) and other workers have found Ca supplements to be beneficial for domestic animals; Coward *et al.* (1938) have found that the addition of Ca and P salts to a poor human dietary fed to rats increased the ash content of the bones; and Aykroyd & Krishnan (1938) have recently reported that improvement of health and growth rate has followed the feeding of Ca lactate to school children in India.

It has also been known for some time that an increase in milk intake causes bodily improvement. The experiments of Corry Mann (1926) demonstrated this very clearly, and Orr & Clark (1930) found that supplementary feeding of school children with milk, either whole or separated, caused a considerable increase in growth and a definite improvement in health.

There would thus appear good reason to suppose that the protective foods owe their special value largely to their Ca and P contents. Sherman & Campbell (1935) showed that the improvement in the nutritional value of synthetic diets caused by adding dried milk could be largely obtained by using an equivalent amount of $CaCO_3$. The experiments reported in the present paper show to what extent such comparison can be made, and the fact that both diets II and III are below the optimal makes comparison possible.

Diets II and III were equally good for inducing bone calcification, tooth formation and reproduction. Diet III appeared, however, to be definitely deficient in growth promoting properties; group III rats grew as well as group II rats only when their parents' diet was optimal; Ca and P salts were sufficient to produce healthy rats but not ones of normal weight. It is not improbable

that this subnormal weight was due to the lack of the protein present in the milk in diet II. The addition of Ca and P to the diet undoubtedly increased the food consumption; it would thus appear that these supplements, besides increasing the nutritional value of the diet, made it more palatable. It is noteworthy that diet III was able to support pregnancy and lactation as well as diet II. In the absence of natural foodstuffs, supplements of Ca and P in salt form would undoubtedly be beneficial to pregnant women. It should not, however, be concluded that Ca and P in salt form are as available as that in milk and green food; this can only be decided by balance experiments.

It would thus appear true that milk and green food owe a great deal of their special nutritional qualities to their Ca and P contents; it is certain that no other elements given alone would have produced these results; and it seems legitimate to ascribe to them a large share in the conversion of "a biologically poor into a biologically good diet".

SUMMARY

1. A group of rats has been fed on a poor human dietary supplemented with milk and green food. A further group has been given the same dietary supplemented with Ca and P as salts, equivalent in amount to that present in the milk and green food. The experiment has been continued over three generations.

2. The diet supplemented with Ca and P salts has been found to be as effective as that containing milk and green food in promoting reproduction, calcification and tooth formation; it has induced growth as effectively in the first generation of rats but is unable to maintain this growth rate as effectively over several generations. It is obvious that Ca and P are of the chief deficiencies in the poor diet. The addition of these elements greatly increases the nutritive value of this diet, but they are inferior in this respect to milk and green food supplements.

3. Under conditions of Ca and P lack, tooth formation is much less adversely affected than is skeletal ossification.

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