LXXX. THE BIOLOGICAL VITAMIN D ASSAY OF LOW-POTENCY MATERIALS WITH SPECIAL REFERENCE TO THE ROLE OF THE MINERAL CONTENT OF THE DIET

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VITAMIN D is biologically determined in this laboratory by a slightly modified form of the line-test described by Morgan [1932]. Such quantities of the test substance as are known, either from the nature of the material or from the results of a preliminary trial, to contain about $\frac{1}{4}$ or $\frac{1}{2}$ I.U. vitamin D are given daily to each of about 10 rachitic rats. The same number of weight- and sex-paired litter-mates of these rats are each given daily doses of $\frac{1}{4}$ or $\frac{1}{2}$ I.U. vitamin D Standard, and the areas of the "lines" of new calcification in each group are compared. Doses of this order, i.e. between about $\frac{1}{4}$ and $\frac{3}{4}$, produce, in the 10-day test-period, degrees of healing in the distal ends of rachitic radii and ulnae most suitable for quantitative assessment; quantities less than $\frac{1}{4}$ unit produce only minute healing, while quantities greater than $\frac{3}{4}$ unit produce massive healing which is difficult to measure and which is too close to the optimal to be critical. To prepare and administer the requisite amounts of high-potency test substances is a simple matter: the oil is diluted with refined arachis oil, so that one or two drops from a calibrated pipette have to be given to each animal daily. But if the expected potency of the test substance is less than 12.5 I.U./g. it has to be fed "neat", and if it is less than 1 I.U./g. the quantity to be fed becomes inconveniently large.

This inconvenience is particularly attendant upon the assaying of most samples of butter and many samples of margarine. Winter butter may contain less than 0.1 I.U./g. [Wilkinson, 1939], while many continental margarines are vitaminized below 1 I.U./g. (in Denmark a lower limit of 0.1 I.U./g. is stipulated by law). If such a sample is to be tested, each rat has to be given about 3 g. fat daily, a quantity which is so large a proportion of the total food consumption (normally a vitamin D test rat eats about 8–10 g. of diet daily) that the animals will not eat it all separately, and it has to be mixed into the diet. Even then it may not be all consumed.

There is an alternative procedure, namely, to extract and feed the vitamin D-containing unsaponifiable fraction of the fat. Until recently there has not been available an efficient and satisfactory method of extraction; the work of Kon & Booth [1934] indicated that some part of the vitamin D of butter fat is too unstable to pass unchanged through the necessary chemical processes. However, Bechtel & Hoppert [1936] described a method of extracting butter fat directly with alcohol and drying the alcoholic extract on a weighed quantity of diet which was then fed in measured amounts to the test animals. We have used this technique for both margarines and butters, but the results were invariably

lower than those from "direct" assays, and there was no means of ascertaining which were the more reliable. An investigation was therefore made into the reliability and limitations of the "direct" method with special reference to the effect, if any, of large quantities of dietary fat on the vitamin D therapy of rat rickets.

Preliminary investigations

The simplest method of testing the reliability of an assay in which the "test substance" animals receive appreciable additions of fat is to carry out a parallel assay in which the control animals receiving vitamin D International Standard also receive equal quantities of a similar, but unvitaminized, fat. For this purpose a vegetable cooking-fat was selected and one portion of it was vitaminized to the extent of approximately 1 I.U./g. by the incorporation of a calciferol concentrate. This was the test substance. In the first assay 5 isogenic pairs of rats were used: one rat of each pair was given 0.5 g. of the vitaminized fat daily and the other 0.5 I.U. vitamin D Standard daily. In the second assay 7 isogenic pairs were used: as before the test substance was given to one half of the rats and the Standard to the other half, but the latter was also given 0.5 g. of the unvitaminized fat per rat per day. The result of the first assay was 1.25 I.U./g. and of the second, 1.1 I.U./g. These two figures are not significantly different.

To confirm directly this indication that 0.5 g. per day of extra fat does not appreciably affect the degree of healing of rickets, we then compared the areas of new calcification in two groups of isogenic pairs of rats, one of which was given vitamin D International Standard alone and the other of which was given the same dose of the vitamin with extra fat. In order to determine how much more fat could be given without disturbing the vitamin D assay, two further groups of rats were included, one of which received 2 g. fat and the other 3 g. fat per rat per day. The results are summarized in Table I.

Table I. *Apparent enhancement of vitamin D potency produced by three levels of addition of an unvitaminized cooking-fat*

No. of pairs	Daily dose of fat to one rat of each pair g.	Daily dose of vitamin D to all rats	✓ Dose ratio:*	Apparent dose given to "fat" rats
				Actual dose given to "no-fat" rats
16	0.5	$\frac{1}{2}$ I.U.		0.935
9	2.0	$\frac{1}{2}$ I.U.		1.044
7	3.0	$\frac{1}{2}$ I.U.		1.568

* Calculated from the standard equation: (Healing)^{0.7} = 54.5 log dose ratio.

These results support the conclusion from the first experiment, for a dose ratio of 0.935 is not significantly different from unity; nor is the ratio 1.044, implying that even 2 g. of supplementary fat daily will not be expected to falsify an assay. The feeding of 3 g. of fat daily, however, has stimulated healing to such an extent that the apparent dose ratio has increased by over 50%. As only 7 pairs were used this figure cannot be regarded as a quantitative index of the effect of the fat; nevertheless the tendency for it to raise the ratio is unmistakable. It is pertinent to record that a few animals which received daily supplements of 2.4 g. of fat but no vitamin D showed slight "spontaneous" healing of rickets—a phenomenon never encountered when normal diets are used.

The finding that the inclusion of 30% or more of an inactive fat in a standard rachitogenic diet will enhance the therapeutic effect of vitamin D and will even induce healing (perhaps better described as apparent healing) in the absence of

the vitamin, admits of either or both of two possible explanations. The first is that the fat *per se* is responsible for the effect by increasing the absorption and utilization of Ca, thereby stimulating ossification. The second is that the effect results from the decreased consumption of the rachitogenic diet itself necessitated by the addition of the fat. The remainder of this paper is mainly devoted to a description of an investigation into the latter possibility.

Experimental diets and technique

The rachitogenic diet (diet 5) in general use in this laboratory is composed of:

		%
Dried meat meal	...	12
Ground unmineralized flaked maize	...	84
Salt mixture	...	4

The relevant analytical figures are:

	Ca %	P %	Protein %
Meat meal	0.125	0.58	78
Maize	0.013	0.28	9

The composition of the salt mixture is:

	%
Calcium carbonate	75
Sodium chloride	15
Magnesium sulphate	7.5
Ferric citrate	2.4
Potassium iodide	0.1

If 38 g. fat are added to every 62 g. diet 5, there will be a corresponding fall in the percentages of maize, meat meal and salt mixture. The actual consumptions of these dietary constituents will fall even more markedly because less of the high-fat, and therefore high-calorie, diet will be eaten than of the normal diet. If the calories/g. in maize be taken as 3, in meat meal as 4 and in fat as 9, and if it be assumed that a rat normally eats sufficient food to supply 30 calories daily, the following quantities of ingredients will be ingested from the two diets:

	Total diet to supply 30 cal.	Meat meal	Maize	Salt mixture	Cooking fat
	g.	g.	g.	g.	g.
Diet 5	10	1.2	8.4	0.4	—
Diet D	5.7	0.42	2.97	0.14	2.17

There is thus a considerably lowered intake of protein and salt mixture, and as the latter supplies nearly all of the Ca—on the high content of which the rickets-producing effect of diet 5, as of Steenbock's diet 2965, depends—it is reasonable to expect that its deprivation is largely responsible for the enhancement of healing.

A series of experiments was then planned in which the degrees of healing produced by 0.5 I.U. daily were compared in groups of rats being fed with diets of varied compositions. For convenience of reference, the compositions of all the diets used are collected in Table II.

In all experiments does only were used. They were maintained on diet 5 from weaning (at 23 days of age), or from the day on which they reached an average weight of 45 g. until the 39th–41st day, when they were transferred to the experimental diets and maintained thereon for the 10-day test-period, at the end of which they were killed and their radii and ulnae removed.

Table II. *Compositions of diets*

Ref.	Cooking-fat %	Meat meal %	Maize %	Salt mixture %	Ca %	P* %	Ca/P* ratio	Protein %
A	33	12	46.5	8.5	2.571	0.200	12.85	13.55
B	33	12	50	5.0	1.522	0.210	7.25	13.86
C	38	12	46	4.0	1.221	0.199	6.15	13.50
D	38	7.5	52	2.5	0.766	0.189	4.05	10.53
E	33	12	53.5	1.5	0.472	0.220	2.15	14.17
5	—	12	84	4.0	1.226	0.305	4.02	16.92
F	—	8.7	88.4	2.9	0.893	0.298	3.00	14.75
G	—	18	80.5	1.5	0.483	0.331	1.46	21.49
H	—	12	87	1.0	0.327	0.314	1.04	17.19

* The P analyses, and consequently the Ca/P ratios, are based on total P and no account has been taken of inorganic, organic, available or unavailable portions.

Experiment 1

This was designed to investigate the effect on the degree of healing of a diet which contained 38 % additional fat but in which the meat meal and salt mixture contents remained unaltered at 12 and 4 % respectively. This was diet C. It was compared with diets 5 and D and a fourth diet, F, which contained no additional fat, but had low meat meal and salt mixture contents, viz. 8.7 and 2.9 % respectively. 27 rats were distributed between these 4 diets so that there were always litter-mate pairs of about the same weight on at least 2 diets. They were all, with the exception of 3 negative controls, given 0.5 I.U. of vitamin D standard per day. The daily food consumptions of all the animals on the experimental diets were measured. The results are assembled in Table III.

Table III. *Average daily food consumptions (F.C.) and areas of new calcification in sq. mm. (H) arranged, except those of the "no dose" rats, horizontally as weight-grouped litter-mates*

Daily dose of vit. D	Diet D			Diet C			Diet F			Diet 5		
	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H
½ I.U.	5392	5.9	434	5393	6.0	258	—	—	—	—	—	—
	—	—	—	5396	5.7	174	5397	8.1	254	5398	—	114
„	5429	6.1	286	5430	6.1	196	—	—	—	—	—	—
	—	—	—	5431	4.9	294	5432	6.5	292	5433	—	310
„	5448	6.3	378	5450	6.5	172	—	—	—	—	—	—
	—	—	—	5460	5.9	180	5461	8.1	232	5464	—	184
	—	—	—	5462	6.0	128	5463	7.3	242	5465	—	156
„	5498	5.0	162	5499	5.6	197	—	—	—	—	—	—
	5501	5.4	378									
„	—	—	—	5502	5.0	208	5504	8.4	358	5503	—	168
No dose	5391	5.4	88	5449	6.1	0	5396	7.8	146	—	—	—

In order to obtain average figures for the 4 dietary groups in the most comparable form from the above table, the following method was used: the group containing the most rats—the diet C group—was taken as a standard against which each of the other groups was compared, for which purpose litter-mate pairs only were used. As an example, the average area of new calcification (i.e. healing) of the rats in the diet F group is 275.6 sq. mm. while the average healing of their 5 litter-mates on diet C is 196.8 sq. mm., the difference being 78.8 sq. mm. Therefore, as the average healing of all the diet C rats is 200.7 sq. mm. the

relative average of the diet F rats is taken as 279.5 sq. mm. This treatment reduces the errors which arise from biological variation of healing responses in different litters and which cannot be taken into account by simple arithmetical averaging. The resultant figures are set out in Table IV.

Table IV. *Relative behaviour of the 4 groups of rachitic rats each dosed daily with $\frac{1}{2}$ I.U. vitamin D standard. All weights expressed as g.*

	Diet D	Diet C	Diet F	Diet 5
No. of animals	5	9	5	5
Average daily food consumption	5.6	5.7	7.9	—
Average daily protein consumption	0.59	0.78	1.17	—
Average daily phosphorus consumption	0.011	0.011	0.024	—
Average daily salts consumption	0.14	0.23	0.23	—
Average daily fat consumption	2.1	2.2	0	—
Average healing in sq. mm.	337	201	279	190

N.B. In preference to Ca intakes, salt mixture intakes only have been considered throughout this work, for although it is highly probable that Ca carbonate is the important variant, nevertheless the presence of the other salts in constant proportions theoretically prohibits the making of any deductions about Ca alone.

The only observation that can be made from this table is that the rats consuming the least quantity of salt mixture—those on diet D—displayed most healing. There is no indication of the existence of a simple relation between cooking-fat consumption and degree of healing. Both undosed animals on the low-salt diets showed some healing.

Experiment 2

This was designed to confirm the findings of Exp. 1 and concomitantly to ascertain to what extent the degree of healing produced on a high-fat diet could be controlled by variation of the intake of salt mixture. The levels of inclusion of salt mixture chosen were 1.5, 5 and 8.5% in a diet containing meat meal,

Table V. *Average daily food consumptions (F.C.) and areas of new calcification in sq. mm. (H) arranged, except those of the "no dose" rats, horizontally as weight-grouped litter-mates*

Daily dose of vit. D	Diet E			Diet B			Diet A			Diet 5		
	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H
$\frac{1}{2}$ I.U.	6629	8.5	930	6630	7.0	146	—	—	—	—	—	—
"	—	—	—	6633	6.8	208	—	—	—	6631	12.1	280
"	6635	7.0	456	6636	7.5	192	6637	7.7	100	—	—	—
"	6673	7.0	342	6671	8.1	296	6672	9.1	240	—	—	—
"	—	—	—	6816	6.7	122	—	—	—	6815	12.7	322
"	6819	6.5	464	6820	7.6	90	—	—	—	—	—	—
"	—	—	—	6751	5.4	356	—	—	—	6750	13.3	202
"	6748	7.0	574	6748	9.1	185	6752	6.6	406	—	—	—
"	—	—	—	6829	6.6	218	—	—	—	6830	10.9	150
"	6834	6.0	336	6832	9.0	130	—	—	—	—	—	—
"	—	—	—	6836	8.2	116	6837	8.8	96	—	—	—
"	8451	7.2	222	8449	6.2	113	8452	7.0	81	8450	8.4	122
"	8460	8.2	391	8464	7.0	167	8462	7.3	39	—	—	—
"	8466	8.4	243	8465	6.8	138	8469	7.6	86	8468	7.6	180
No dose	6632	6.1	522	6638	6.9	0	—	—	—	6634	7.9	0
"	6818	5.7	177	—	—	—	—	—	—	6817	10.6	0
"	6833	4.4	242	6835	6.1	0	—	—	—	6828	8.9	0
"	8467	5.9	190	—	—	—	—	—	—	6831	9.0	0
"	8545	6.9	110	—	—	—	—	—	—	—	—	—
"	8581	5.8	158	—	—	—	—	—	—	—	—	—

maize and 33 % cooking-fat. These were designated diets E, B and A respectively. Control animals were maintained on diet 5 and this time all food consumptions, including those of the diet 5 animals, were measured. On each diet a few animals were maintained without vitamin D doses. In all other particulars the experimental details were the same as before. The data from this experiment, in which 48 rats were used, are collected in Table V.

The comparative figures for the several dietary groups, obtained as explained previously, have been arranged in Table VI.

Table VI. *Relative behaviour of the 4 groups of rachitic rats, each dosed daily with 0.5 I.U. vitamin D. All weights expressed as g.*

	Diet E	Diet B	Diet A	Diet 5
No. of animals	9	14	7	6
Average daily food consumption	7.0	7.3	7.1	11.7
Average daily protein consumption	0.99	1.01	0.97	1.98
Average daily phosphorus consumption	0.015	0.015	0.014	0.036
Average daily salts consumption	0.11	0.37	0.61	0.47
Average daily fat consumption	2.3	2.4	2.4	0
Average healing in sq. mm.	455	177	154	194

Again it appears that salt mixture intake and degree of healing are the important variants. A salient feature of the results is the massive healing displayed by the rats which were consuming the lowest level of salt mixture. The average daily salt mixture consumption of these rats, 0.11 g., is about one-fifth of the normal and the areas of new calcification were so large that accurate assessment was difficult. Moreover, the 3 undosed rats receiving diet E showed considerable "spontaneous" healing. The figures in Table VI do not suggest that the excessive dietary fat has any appreciable direct effect on the healing. It will have been observed, however, that the tabulated results of the last two experiments indicate the existence of some correlation between healing and one other factor, viz. the Ca/P ratios of the diets. As the P contents do not vary within wide limits, it is to be expected that the Ca/P ratios will closely follow the Ca contents: this aspect will be considered in detail later.

Experiment 3

The next experiment was on a smaller scale. Its object was to discover whether spontaneous healing of rickets could be effected by feeding a diet containing very small quantities of salt mixture but no additional fat. For this purpose diet H was made up. As the animals would eat more of this diet than of the high-fat diet E, the salt mixture was included at only 1 %. Some of the rats received vitamin D and some received none. Table VII contains the relevant data. Food consumptions were not recorded.

Table VII. *Production of "spontaneous" healing of rickets by the feeding of a low-salt diet for 10 days*

	Diet 5 (undosed)	Diet 5 ($\frac{1}{2}$ I.U./day)	Diet H (undosed)	Diet E (undosed)
No. of animals	2	2	3	3
Average healing in sq. mm.	0	36	82	117

Extensive healing has taken place on the low-salt diet in the absence of either fat or vitamin D. It is impossible, with so few rats, to state whether or not the difference between the healings on diets H and E is significant. A more elaborate comparison of the two diets was then conducted.

Experiment 4

To compare with diets H, E and 5, a new diet, diet G, was introduced, which contained no added fat, only 1.5 % salt mixture, but 18 % meat meal; its purpose was to determine the effect of increasing the protein intake of the animals. The conduct of the experiment conformed closely to that of the three main experiments reported above, and the results are presented in a similar form in Table VIII.

Table VIII. *Average food consumptions (F.C.) and areas of new calcification in sq. mm. (H) arranged, except those of the "no dose" rats, horizontally as weight-grouped litter-mates*

Daily dose of vit. D	Diet E			Diet H			Diet G			Diet 5		
	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H	Rat no.	F.C.	H
$\frac{1}{2}$ I.U.	—	—	—	8538	9.4	480	—	—	—	8537	10.6	125
"	—	—	—	8539	9.7	480	—	—	—	8541	10.9	150
"	—	—	—	8540	10.9	463	—	—	—	8542	10.3	166
"	—	—	—	8602	8.2	530	8598	9.0	320	8601	8.1	173
"	—	—	—	—	—	—	8600	9.6	451			
"	—	—	—	8614	11.6	444	8616	11.4	539	8615	10.8	257
"	—	—	—	8617	11.6	537						
"	—	—	—	8619	8.4	543	8618	10.2	442	—	—	—
"	—	—	—	8644	10.2	492						
"	8648	7.5	411	8645	10.6	586	—	—	—	—	—	—
"	8690	8.2	416	8689	10.6	332	—	—	—	—	—	—
"	—	—	—	8692	8.1	413	—	—	—	8691	9.9	246
"	8702	7.7	423	8701	10.4	410	—	—	—	—	—	—
"	8703	7.5	407				—	—	—	—	—	—
"	8731	8.9	372	8733	11.3	466	—	—	—	—	—	—
"	8734	8.5	353	8736	10.3	462	—	—	—	—	—	—
"	8768	7.3	418	8769	9.4	364	—	—	—	—	—	—
No dose	8730	6.3	163	8599	7.4	172	8597	6.7	142	8643	10.7	0
"	8732	6.3	211	8770	8.2	326	—	—	—	—	—	—
"	8735	6.9	130	—	—	—	—	—	—	—	—	—

By the usual method the group averages from these data have been collected in Table IX.

Table IX. *Relative behaviour of the 4 groups of rachitic rats, each dosed daily with 0.5 I.U. vitamin D. All weights expressed as g.*

	No. of animals	...	Diet E	Diet H	Diet G	Diet 5
			7	15	4	6
Average daily food consumption			7.6	10.1	10.8	10.4
Average daily protein consumption			1.07	1.73	2.33	1.76
Average daily phosphorus consumption			0.017	0.032	0.036	0.032
Average daily salts consumption			0.114	0.10	0.16	0.42
Average daily fat consumption			2.5	0	0	0
Average healing in sq. mm.			431	467	406	181

It will be noted that neither the protein nor the fat consumption has any manifest bearing on the degrees of healing.

DISCUSSION

All the experiments that have been described have yielded consistent results and the three major experiments (nos. 1, 2 and 4) in particular appear to be mutually supporting. In order to obtain a composite picture of the results they

have been re-tabulated below in dietary groups placed in order of the average degree of healing of rickets.

Table X. *Collected data concerning healing of rickets and diet. (All rats dosed $\frac{1}{2}$ I.U. vitamin D for 10 days; all weights expressed as g.)*

Exp. no.	No. of rats	Diet	Av. healing in sq. mm.	Av. daily consumption of				Ca/P ratio of diet
				Salt mixture	Phosphorus	Protein	Cooking-fat	
4	15	H	467	0.10	0.032	1.7	0	1.04
2	11	E	455	0.10	0.015	0.99	2.3	2.15
4	7	E	431	0.11	0.017	1.1	2.5	2.15
4	4	G	406	0.16	0.036	2.3	0	1.46
1	5	D	337	0.14	0.011	0.59	2.1	4.05
1	5	F	279	0.23	0.024	1.2	0	3.00
1	9	C	201	0.23	0.011	0.78	2.2	6.15
2	6	5	194	0.47	0.036	2.0	0	4.02
4	6	5	181	0.42	0.032	1.8	0	4.02
2	14	B	177	0.36	0.015	1.0	2.4	7.25
2	9	A	154	0.60	0.014	0.97	2.4	12.85

Of the five dietary variants listed, three, namely, phosphorus, protein and fat, are obviously unrelated to healing, while the other two, salt mixture intake and Ca/P ratio tend to increase with decrease of healing. The variation of the Ca/P ratio is of approximately the same order as that of the salt mixture intake; and this is to be expected because (a) the phosphorus, being wholly supplied by the maize and meat meal, does not vary widely, and (b) salt mixture intakes are not markedly disproportional to salt mixture contents of the diets. Although from the practical standpoint the ratio is unimportant, and will not therefore be taken into consideration, theoretically it merits close attention [cf. Bethke *et al.* 1932; Shohl, 1936; 1937]. To investigate the true role of the Ca/P ratio, diets with constant Ca contents and variable P contents must be included; moreover, allowance must be made for the presence of such unavailable forms of P as inositolhexaphosphate [Bruce & Callow, 1934, 2]. In the preceding Tables total P only has always been given and no account has been taken of the availability of the element. Consequently the Ca/P ratios given are probably lower than the more important Ca/available-P ratios.

For the purpose of graphical presentation of the relation between the two variants the collected results were divided into 4 groups, which were:

- (i) rats receiving $\frac{1}{2}$ I.U. daily and supplementary fat,
- (ii) rats receiving $\frac{1}{2}$ I.U. daily and no supplementary fat,
- (iii) and (iv) the undosed rats with and without supplementary fat respectively.

The healings of each group were then arranged in order of intensity and marked off into subgroups each representing about 8 rats. Average healing and salt intake were calculated for each subgroup and the resulting figures plotted. Necessarily it was impossible to take into consideration weight- or isogenic-grouping, but the large number of animals used (there were 131 altogether, including 20 odd "unpaired" rats on the several diets which could not be incorporated in the separate experimental results) would cancel any errors due to variability of litter responses. The resultant averages were used to construct the curves in Fig. 1, which clearly illustrate both the lack of effect of the added fat and the relation of rickets healing to salt mixture intake with and without the presence of vitamin D.

The requisite adjustment of salt mixture intakes in an assay involving the feeding of large quantities of fat can be carried out in any of three ways:

- (a) indirectly by equalization of fat percentages in all diets,
- (b) directly by the equalization of the salt mixture in the diets as parts per anticipated daily consumption,
- (c) directly by the equalization of the salt mixture in the diets as parts per calorie.

Fig. 1. The small figures indicate the number of rats represented by each point.

Because the primary object of this investigation was to determine the best method of assaying low-potency butters and margarines, all experiments have been based on the standard assay technique with its 16–18 day rachitogenic period on “diet 5”. The ancillary derivation of the relation between degree of healing and salt mixture intake cannot therefore be directly compared with similar derivations made by other workers who have usually fed their experimental diets from weaning. It is interesting to note, however, that Querido [1935] fed to rats a diet containing 0.35 % Ca and 0.35 % P (i.e. similar to our diet H, which gave very heavy healing) and found that it was non-rachitogenic even in the absence of vitamin D. Bruce & Callow [1934, 1] showed that the addition of available P to a high Ca/P diet markedly increased the therapeutic efficacy of vitamin D dosing.

One aspect of rickets has been left untouched, viz. the composition of the bone ash. The effect of various salt mixtures on this will be examined in a subsequent publication.

SUMMARY

Experiments are described in which rachitic rats have been dosed with equal quantities of vitamin D in conjunction with various levels of the standard rachitogenic salt mixture, and, in some cases, with large quantities of fat. It has been found:

(1) that, under the conditions of experimentation in this laboratory, the presence of excess fat in the diet influences the degree of new calcification produced by vitamin D only indirectly by lowering the consumption of salts. In assay practice this means that low-potency butter and margarines can be

accurately tested only by equalization of the salt mixture intakes of test substance and "standard" rats;

(2) that the healing of rickets produced by vitamin D, as measured by the line test, is inversely proportional to the intake of salt mixture of which Ca is probably the influential constituent, and that a very low salt mixture intake will produce healing in the absence of vitamin D.

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