52. A Study of the Antirachitic Effect of Fat on Rats receiving High Calcium-Low Phosphorus Rachitogenic Diets*

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Several years ago Kon & Booth [1933; 1934 *a, b*] found that the antirachitic activity of butter as measured on rats could not be quantitatively concentrated in the non-saponifiable residue by a method which yielded complete recovery from more potent sources of vitamin D, such as a solution of irradiated ergosterol in olive oil (International Standard for vitamin D), or cod-liver oil. This finding has since been abundantly confirmed [Bechtel & Hoppert, 1936; Boer, 1938; Gridgeman, Lees & Wilkinson, 1940].

Kon & Booth [1933; 1934 *a, b*] also failed to concentrate quantitatively the vitamin D activity of butter by alcoholic extraction after the method of Zucker [1922-3; 1925; 1928]. Though Bechtel & Hoppert [1936] have recently advocated the use of this method for the estimation of vitamin D in butters of low potency, the experience of other workers has been uniformly negative [Wallis, 1937; 1939; Gridgeman, Lees & Wilkinson, 1939; Wilkinson, 1939; Boer, 1939].

Kon & Booth [1933; 1934 *a, b*] suggested that the unorthodox behaviour of the vitamin D activity of butter could be explained by the fat itself having an antirachitic effect on rats receiving high Ca-low P rachitogenic diets. They gave experimental support to this contention by showing that olive oil and also fatty acids from butter and lard, all presumably free from vitamin D, exerted a definite antirachitic effect. Several workers, among them Knudson & Floody [1940] and Jones [1940], who has reviewed the earlier work on the subject, have since shown that vitamin D-free fats have an antirachitic action. It is generally agreed that this effect is due to some direct action of the fat on the Ca and P metabolism of the rat, though the mode of action is as yet by no means clear.

Gridgeman *et al.* [1939], who also noticed the calcifying effect of fat, were of the opinion, however, that '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... 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'. They suggest that 'low-potency butter and margarine can be accurately tested only by equalization of the salt mixture intakes of test substance and "standard" rats'. The salt mixture used by these authors contained 75% of CaCO_3 but no P, and it is clear that their conclusions and advice are based on a misunderstanding and are erroneous. The addition of fat to a diet does not, as Gridgeman *et al.* [1939] appear to assume, affect preferentially any one component of a ration, and would lower the intake of Ca from their salt mixture to no greater degree than the intake of P from the rest of the diet. The absolute amounts of both these components would be decreased but their ratio remain unchanged. It has been shown by Brown, Shohl, Chapman, Rose & Sauerwein [1932] and Shohl [1936] that at a given Ca : P ratio rickets is intensified by a decrease in the level of these two elements. If the only function of fat were to cut down the intake of Ca and P by acting as a diluent and also by increasing the calorific value of the diet, its addition would lead, if anything, to more severe rickets.

* Read in part before the Biochemical Society, 16 March 1940 [Henry & Kon, 1940].

In the experiments on the antirachitic effects of fat so far reported by ourselves and by other workers natural fats, free, mixed or isolated fatty acids, or Ca soaps were used. It is conceivable that the natural fats contained some vitamin D, and it might be argued that the effects of free fatty acids or their soaps on Ca metabolism are different from those of neutral fats. For this reason we have carried out numerous tests with triglycerides resynthesized from fatty acids from which the non-saponifiable matter had been removed as thoroughly as possible. We have also done experiments in which due allowance was made for the change in Ca and P levels of a rachitogenic diet which follows the addition of fat. This can be done by giving to one group of animals the diet containing fat and to another a similar diet in which the fat is replaced by an equicaloric quantity of starch and thus equalizing the calorie intakes of the two groups. The same result can also be achieved by compounding a synthetic rachitogenic ration in which, to avoid differences in availability, all the P is present as inorganic phosphate, and by feeding it as such or with the addition of fat and of extra Ca and P so that equicaloric quantities of both diets would again supply the same levels and ratios of Ca and P. We used both these methods. Finally, metabolic tests were planned to obtain information on the mode of action of fat. Some of the experiments took place several years ago but have not previously been published.

EXPERIMENTAL

1. Comparison of the effects on calcification in rats of starch and fat when added as isocaloric diluents to a rachitogenic ration

A. Experiments with Steenbock's 2965 diet

(a) *Diets.* Three diets were used. Diet 28 A was identical with the 2965 diet of Steenbock [Steenbock & Black, 1925], but an extra 0.35 g. CaCO_3 was added to 100 g. of the diet. The composition and analysis of diets 124 and 125 are given in Table 1.

Table 1. *Composition and analysis of the diets* used in the various experiments*

Diet no.	Equicaloric quantities	% Ca	% P	Ca/P	% moisture
Exp. 1 A (diets fed dry)					
28 A	140 g.	1.306	0.323	4.0	10.75
124	80 g. 28 A + 52 g. rice starch	0.820	0.213	3.9	11.64
125	80 g. 28 A + 20 g. ground-nut oil no. 1	1.052	0.261	4.0	8.26
Exp. 1 B (diets fed dry)					
120	140 g.	1.223	0.247	4.9	14.88
126	80 g. 120 + 52 g. rice starch	0.734	0.167	4.4	13.90
127	80 g. 120 + 20 g. ground-nut oil no. 1	0.961	0.203	4.7	11.52
Exp. 2 (diets fed mixed with water)					
124	80 g. 28 A + 52 g. rice starch	0.820	0.213	3.9	11.64
129	80 g. 28 A + 20 g. resynthesized triglycerides of ground-nut oil no. 1	1.089	0.256	4.3	8.35
130	80 g. 28 A + 20 g. resynthesized triglycerides of butter-fat	1.085	0.252	4.3	8.48
Exp. 4 (diets fed dry)					
131	130 g.	1.352	0.104	13.0	9.24
132	75.6 g. 131 + 21.9 g. ground-nut oil no. 1 + 1.85 g. CaCO_3 + 0.65 g. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	1.777	0.136	13.1	7.35
Exp. 5 A (diets fed dry)					
142	80 g. 141 + 20 g. ground-nut oil no. 2	0.064	0.252	0.25	9.98
143	80 g. 141 + 52 g. maize starch	0.055	0.197	0.28	12.68
Exp. 6 (diets fed mixed with water)					
127 A	80 g. 120 + 20 g. ground-nut oil no. 2	0.9951	0.2048	4.8	9.18
144	80 g. 120 + 52 g. maize starch + N.S.R. of 20 g. ground-nut oil no. 2	0.7511	0.1846	4.1	12.09

* The diets are fully described in the text.

Table 2. *Exps. 1A and 2. Data for rats receiving the rachitogenic diet of Steenbock & Black [1925] with the addition of starch or fats*

Litter no.	Wt. of rat (g.)			Diet intake g.	Ca intake g.	P intake g.	% bone ash	Wt. of rat (g.)			Diet intake g.	Ca intake g.	P intake g.	% bone ash
	Initial	Gain						Initial	Gain					
Diet 28 A (Steenbock & Black's rachitogenic diet)														
1	58	24	280-16	3-659	0-905	0-905	28-4	58	21	261-86	2-146	0-557	32-2	33-3
2	57	21	263-08	3-436	0-850	0-850	34-4	57	9	234-20	1-920	0-499	32-6	31-5
3	58	26	279-85	3-655	0-904	0-904	33-2	59	17	266-32	2-184	0-567	31-8	37-0
4	57	22	275-64	3-600	0-890	0-890	31-2	60	17	262-32	2-151	0-559	31-1	34-7
5	59	34	283-28	3-700	0-915	0-915	28-2	58	25	267-46	2-193	0-570	30-4	33-7
6	63	5	225-76	2-948	0-729	0-729	32-1	59	8	227-72	1-867	0-485	32-8	35-8
7	63	23	263-09	2-436	0-850	0-850	32-0	60	16	253-72	2-081	0-540	34-0	32-2
8	63	17	275-49	3-598	0-890	0-890	34-3	61	16	257-27	2-110	0-548	39-4	38-4
Mean	59-8	21-5	268-27	3-379	0-867	0-867	31-7	59-0	16-1	255-08	2-082	0-541	31-8	34-6
Exp. 1A (diets fed dry)														
Diet 124 (diet 28 A + starch)														
Diet 125 (diet 28 A + ground-nut oil)														

	Exp. 2 (diets fed mixed with water)										Diet 130 (diet 28 A + butter triglycerides)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																			
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Table 4. *Exp. 1B. Data for rats receiving the rachitogenic diet of Gridgeman *et al.* [1939] alone or with added starch or fat. Diets fed dry*

Litter no. and sex	Wt. of rat (g.)			Diet intake g.	Ca intake g.	P intake g.	% bone ash	Wt. of rat (g.)			Diet intake g.	Ca intake g.	P intake g.	% bone ash
	Initial	Gain						Initial	Gain					
Diet 120 (rachitogenic diet of Gridge-man <i>et al.</i>)														
15, ♂	59	51					29.5	60	31					30.7
16, ♂	60	43	1111.0	13.588	2.744		25.5	61	23	830.0	6.092	1.386	24.2	24.6
2, ♂	59	52					35.3	58	29				23.6	30.7
17, ♂	60	40					28.0	58	29				28.7	32.9
18, ♂	59	30	979.0	11.973	2.418		29.7	60	25	939.0	6.892	1.568	24.8	26.2
19, ♂	59	39					27.0	58	30				25.8	28.8
16, ♀	58	32					30.8	60	23				29.0	32.5
18, ♀	60	34	986.5	12.065	2.437		29.5	59	24	847.0	6.217	1.414	28.8	31.4
20, ♀	58	37					28.1	61	23				27.7	32.4
5, ♀	59	27					30.6	59	32				27.9	36.5
21, ♀	60	45	949.5	11.612	2.345		31.0	56	26	908.0	6.665	1.516	28.2	33.3
21, ♀	55	47					29.8	61	31				28.4	27.8
Mean	58.8	39.8	335.5	4.103	0.829		29.6	59.3	27.2	293.6	2.156	0.490	26.8	30.7

Litter no. and sex	Wt. of rat (g.)			Diet intake g.	Ca intake g.	P intake g.	% bone ash	Wt. of rat (g.)			Diet intake g.	Ca intake g.	P intake g.	% bone ash
	Initial	Gain						Initial	Gain					
Diet 127 (diet 120 + fat)														
15, ♂	59	51					29.5	60	34					30.7
16, ♂	60	43	1111.0	13.588	2.744	2.744	25.5	61	23	830.0	6.092	1.386	24.2	24.6
2, ♂	59	52					35.3	58	29				23.6	30.7
17, ♂	60	40					28.0	58	29				28.7	32.9
18, ♂	59	30	979.0	11.973	2.418	2.418	29.7	60	25	939.0	6.892	1.568	24.8	26.2
19, ♂	59	39					27.0	58	30				25.8	28.8
16, ♀	58	32					30.8	60	23				29.0	32.5
18, ♀	60	34	986.5	12.065	2.437	2.437	29.5	59	24	847.0	6.217	1.414	28.8	31.4
20, ♀	58	37					28.1	61	23				27.7	32.4
5, ♀	59	27					30.6	59	32				27.9	36.5
21, ♀	60	45	949.5	11.612	2.345	2.345	31.0	56	26	908.0	6.665	1.516	28.2	33.3
21, ♀	55	47					29.8	61	31				28.4	27.8
Mean	58.8	39.8	335.5	4.103	0.829	0.829	29.6	59.3	27.2	293.6	2.156	0.490	26.8	30.7

Diet 125 consisted of 80 parts of diet 28 A and 20 parts of refined deodorized semi-hardened ground-nut oil (i.v. 71; m.p. 28–31°); diet 124 had 80 parts of diet 28 A and 52 parts of rice starch. On the calorie basis and when due allowance was made for moisture 20 g. of fat were equivalent to 52 g. of rice starch and to 60 g. of diet 28 A; thus 100 g. of diet 125 was equivalent to 132 g. of diet 124 and to 140 g. of diet 28 A.

(b) *Rats.* Rats were prepared for all experiments described here as follows. On the 16th day of lactation butter was removed from the stock diet [Folley, Ikin, Kon & Watson, 1938] of the mothers and the young were weaned when weighing from 55 to 64 g. In all experiments, except no. 6, all animals were given 1 g. carrot a week as a source of carotene. The experiments lasted 5 weeks. At the end of this time the animals were killed by coal gas and the ash content of the femora and humeri determined [Kon & Henry, 1935]. In the first experiment eight groups of three littermate male rats were allocated by lot to the three diets and placed in individual cages. The diets were fed dry and the calorie intake was equalized from day to day between littermates. Tap water was given to drink.

Table 3. *Statistical treatment* of differences in bone ash observed in the various experiments and of Ca and P retentions in Exp. 6*

Difference tested between diets	Numerical difference	Standard error of mean	P†	Difference tested between diets	Numerical difference	Standard error of mean	P†
Exp. 1 A: % bone ash				Exp. 4: % bone ash			
124–28 A	+0.1	±0.96	1:1	132–131	+3.9	±0.59	1:3030
125–124	+2.8	±1.23	1:18	Exp. 5 A: % bone ash			
125–28 A	+2.9	±0.99	1:42	143–142	+0.6	±0.72	1:2
Exp. 1 B: % bone ash				Exp. 6: % bone ash			
120–126	+2.8	±0.95	1:76	127 A–144	+3.4	±0.95	1:86
127–126	+3.9	±0.79	1:2500	% Ca retention			
127–120	+1.1	±0.95	1:4	127 A–144	+7.64	±3.78	1:11
Exp. 2: % bone ash				% P retention			
129–124	+5.0	±0.90	1:1250	127 A–144	+10.92	±3.77	1:36
130–124	+4.9	±0.73	1:3030				
129–130	+0.1	±0.65	1:1				

* The *t*-test of 'Student' [1908; 1925] was used in all comparisons.

† *P* = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

(c) *Results.* The results are given in Tables 2 and 3. They show that the intakes of calories, Ca and P were very nearly the same on diets 124 and 125, yet that the rats receiving the diet containing fat had apparently better calcified bones. The difference is almost, though not quite, significant according to the customary statistical standards. There was no difference in calcification between the rats consuming the ordinary and the starch-diluted rachitogenic diet.

B. *Experiments with the diet of Gridgeman et al. [1939]*

To put the interpretation of the fat effect advanced by these authors to a direct test experiments were carried out with their diet (diet 120, Table 1). Rice starch (diet 127) and refined semi-hardened ground-nut oil (diet 126) were again used as diluents. 12 rats (6 ♂♂ and 6 ♀♀) received each diet and comparisons were all between littermates. The intakes were not equalized but rats were kept three in a cage and the food consumption in each cage was recorded. The results are given in Tables 3 and 4. Here again the difference between the diluents is quite marked, and though rickets was pronounced in all groups the diet containing fat brought about a significantly better calcification than the diet containing starch. This time the bones of the rats receiving fat were little better than those of the animals given the undiluted rachitogenic ration.

2. *Comparison of the effects on calcification in rats of starch and of resynthesized triglycerides when added as isocaloric diluents to a rachitogenic ration*

This experiment was similar to the first, but instead of ground-nut oil resynthesized triglycerides prepared from it and also from butter were used.

(a) *Diets.* The composition and analysis of the diets are given in Table 1. Diet 129 contained resynthesized triglycerides of ground-nut oil and diet 130 those of butter. In other respects these diets were similar to diet 125.

(b) *Method of preparation of triglycerides.* Ground-nut oil from the same batch as used in the previous experiments and butter fat churned in October from the milk of the Institute's herd were saponified by the short method as described by Kon & Booth [1933]. After exhaustive extraction of the non-saponifiable residue with six changes of ether the fatty acids were prepared as described by Kon & Booth [1934 b]. They were then converted into triglycerides by the following method: from 300 to 600 g. of fatty acids were mixed in a 10% excess with the calculated amount of glycerol. Camphor- β -sulphonic acid was added as catalyst to the extent of 0.22% of the weight of the fatty acids. The mixture was then heated in an oil bath to 160° for 2 hr. under atmospheric pressure, then for 3 hr. in the vacuum of a water pump, and finally for 1–2 hr. connected through traps to a 'Hyvac' vacuum pump.

After cooling the reaction mixture was dissolved in 2½ vol. of ether and was washed with 100–200 ml. portions of 10% aqueous K_2CO_3 until no more fatty acids were removed. After 2–3 washings with water the solution was dried over anhydrous Na_2SO_4 and the ether was then removed. The yield of triglycerides was about 85% by weight of the fatty acids used. The triglycerides made from ground-nut oil had an iodine value of 69 as compared with 71 for the original fat. For butter the values were 42 and 44 respectively. The acid value of the resynthesized material varied from 0.22 to 0.44. We realize that the procedures as applied to butter must have resulted in some loss of volatile fatty acids.

(c) *Rats.* Eight groups of three littermate male rats were used and the experimental conditions were as in Exp. 1 A; to minimize scattering, however, the diets were mixed with three-quarters of their weight of water. The results are given in Tables 2 and 3. They confirm those previously obtained and show the unmistakable calcifying effect of fat devoid of vitamin D. The differences are statistically significant (Table 3). The P content of the starch diet was slightly higher than that of equicaloric amounts of the fat diets. This was due to traces of P present in starch and would, if anything, increase the bone ash content of the rats receiving this diet. There was no difference in antirachitic effect between the fats derived from butter and from ground-nut oil, showing that vitamin D had been quantitatively extracted from the former. It will be noticed that the ash content of the bones was higher in this experiment than in corresponding groups in the previous one. This may have been due to biological variations; it is probable, however, that feeding the diet wet was the cause of the difference [Booth, Henry & Kon, 1935]. Separate experiments showed that the butter-fat itself, when incorporated in the same proportion in the basal diet, produced in a group of eight female rats a bone ash of 49.1%. At the same time its non-saponifiable residue fed at a corresponding level to eight female rats receiving the basal diet gave a bone ash of 44.8%, while eight females receiving the unsupplemented diet had 32.6% ash in their bones. These diets were fed dry.

3. *The calcifying effect of triglycerides resynthesized from various natural fats and oils*

This experiment was done in 1934 and smaller numbers of rats were used.

(a) *Diets.* Diet 28 A was fed to all rats. In addition, to some animals resynthesized triglycerides were given by mouth to the extent of 1 g. daily, roughly corresponding to 12.5% of the diet; otherwise the experimental conditions were as already described. The

synthetic triglycerides were prepared very much in the same way as in the second experiment. The following oils were used: olive oil (Crosse and Blackwell, first pressing), coconut oil, deodorized soya bean oil, raw linseed oil (all three from British Oil and Cake Mills, Hull), home-rendered lard [Kon & Booth, 1934 *b*] and butter (N.I.R.D., churned in January).

(b) *Results.* The results are given in Table 5. Without exception all synthetic fat diets gave better calcification than the diet without added fat, but in view of the small number of animals used caution must be used in interpreting results. It would be unwise

Table 5. *Exp. 3. Data for rats receiving the rachitogenic diet of Steenbock & Black [1925] ad lib, supplemented with 1 g. daily of the resynthesized triglycerides of various natural fats and oils. Diet fed dry*

Litter no. and sex	Wt. of rat g.		% bone ash	Litter no. and sex	Wt. of rat g.		% bone ash	Litter no. and sex	Wt. of rat g.		% bone ash	Litter no. and sex	Wt. of rat g.		% bone ash
	Initial	Gain			Initial	Gain			Initial	Gain			Initial	Gain	
Diet 28A				Diet 28A + coconut oil				Diet 28A + lard				Diet 28A + olive oil			
I, ♀	58	28	32.0	I, ♂	58	22	32.6	I, ♂	59	15	35.5	I, ♂	58	22	31.6
II, ♂	57	34	33.5	II, ♀	62	22	34.9	II, ♀	60	26	38.4	II, ♀	63	25	35.2
IV, ♀	60	24	32.4	III, ♀	62	24	32.7	III, ♂	61	29	37.3	III, ♂	58	27	30.7
IV, ♂	61	29	31.0	IV, ♂	61	6	37.5	IV, ♀	60	11	43.4	IV, ♂	58	14	37.3
Mean	59.0	28.8	32.2	Mean	60.8	18.5	34.4	Mean	60.0	20.3	38.7	Mean	59.3	22.0	33.7
Diet 28A + linseed oil				Diet 28A + soya oil				Diet 28A + butter							
I, ♀	59	9	38.7	I, ♂	59	13	34.6	I, ♀	62	15	38.2				
II, ♂	59	18	36.0	II, ♀	60	18	37.3	II, ♂	58	24	35.1				
III, ♂	60	18	33.7	III, ♂	62	16	35.5	III, ♂	59	28	34.7				
IV, ♀	62	1	38.1	IV, ♂	60	3	39.1	IV, ♀	60	14	42.9				
Mean	60.0	11.5	36.6	Mean	60.3	12.5	36.6	Mean	59.8	20.3	37.7				

Table 6. *Exp. 4. Data for rats receiving a synthetic rachitogenic diet with or without added fat. Diets fed dry*

Litter no.	Wt. of rat g.		Diet intake g.	Ca intake g.	P intake g.	% bone ash	Wt. of rat g.		Diet intake g.	Ca intake g.	P intake g.	% bone ash													
	Initial	Gain					Initial	Gain																	
Diet 131													Diet 132 (+ fat)												
15	60	9	789.24	10.671	0.821	29.0	62	17	741.49	13.176	1.008	34.2													
9	60	14				26.5	60	17				31.2													
20	58	5				27.0	59	27				30.4													
7	58	10				25.8	58	17				31.3													
10	60	14				27.5	59	21				31.5													
22	60	6	799.09	10.804	0.831	31.1	63	19	842.27	14.967	1.145	31.5													
14	59	10				28.2	58	26				33.1													
14	59	12				28.7	60	24				31.6													
Mean	59.3	10.0	198.54	2.684	0.207	28.0	59.9	21.0	197.97	3.518	0.269	31.9													

to speculate as to the effects due to differences in constitution between the fats. The diets were not balanced for Ca and P but rats receiving fats were compared with rats given the undiluted rachitogenic ration. As said above, this would, if anything, tend to counteract the fat effect.

4. The calcifying effect of fat when included in a rachitogenic diet made of purified constituents

(a) *Diets.* The rachitogenic diet (no. 131, Table 1) consisted of dried, heat-coagulated egg white, 13.7%; castor sugar, 9.17%; rice starch, 66.10%; semi-hardened ground-nut oil, 4.99%; Ca and P-free salt mixture [Henry & Kon, 1937], 2.69%; $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, 0.29%; and CaCO_3 , 3.00%. The analysis of this diet and the composition and analysis

Litter no.	Wt. of rat		Diet intake g.	Ca intake g.	P intake g.	% bone ash	Wt. of rat		Diet intake g.	Ca intake g.	P intake g.	% bone ash
	g.						g.					
	Initial	Gain					Initial	Gain				
			Diet 142 (+ fat)						Diet 143 (+ starch)			
23	58	26	200.90	0.1283	0.5063	40.3	56	25	258.14	0.1420	0.5075	43.3
24	58	32	227.98	0.1456	0.5745	36.9	60	32	298.60	0.1642	0.5870	40.2
25	57	35	232.98	0.1489	0.5871	40.1	56	29	281.70	0.1549	0.5538	42.2
26	57	27	196.36	0.1255	0.4948	42.5	58	24	259.03	0.1425	0.5093	44.1
27	57	24	165.77	0.1059	0.4177	42.8	58	20	224.68	0.1236	0.4417	39.2
27	62	39	179.48	0.1148	0.4523	41.0	58	40	237.57	0.1307	0.4671	40.2
28	58	9	198.17	0.1267	0.4994	44.9	58	10	260.86	0.1435	0.5129	43.3
28	62	15	241.96	0.1545	0.6097	43.4	60	13	318.44	0.1751	0.6261	44.2
Mean	58.6	25.9	205.45	0.1313	0.5177	41.5	58.0	24.1	267.38	0.1471	0.5257	42.1

(b) *Rats*. Eight pairs of littermate female rats were used. The calorie intakes were equalized within pairs as already described. The diets were fed dry.

(c) *Results*. The results are given in Tables 3 and 7. This time there is no difference between calcifications on the two diets. The Ca content of the starch diet was slightly higher than that of an equicaloric amount of the fat diet, owing to presence of traces of Ca in starch which became noticeable at this low level of Ca intake. It is very doubtful whether this could in any way account for the lack of difference between the two diets. It will be remembered that a similar difference in the P levels between diets 124 and 130 (Table 2) did not prevent the manifestation of the fat effect with high Ca-low P diets.

B. Lack of improvement in calcification following the addition of fatty acids to a high P-low Ca rachitogenic diet

This experiment was carried out in 1934. A diet similar to Steenbock's 2965 but with the Ca : P ratio reversed was made by omitting the 3% CaCO_3 and adding instead 1.45 parts of $\text{CaH}_4(\text{PO}_4)_2$, H_2O and 3.33 parts of Na_2HPO_4 , $12\text{H}_2\text{O}$ to 97 parts of the basal ration (diet 44). The Ca : P ratio was 1 : 3.99. The fatty acids diet (diet 44 A) was made by mixing 7 parts of this diet with 1 part of fatty acids of home-rendered lard [Kon & Booth, 1934b]. Both diets were fed *ad lib.*, 4 pairs of littermate rats being used. The results are shown in Table 8 and can be contrasted with the results of feeding the same fatty acids combined with the orthodox 2965 diet of Steenbock reported by Kon & Booth [1934b]. They certainly lend support to the findings described by us under the preceding caption.

Table 8. *Exp. 5B. Data for rats receiving a low Ca-high P diet alone or with added fat. Diets fed dry*

Litter no. and sex	Wt. of rat (g.)		% bone ash	Wt. of rat (g.)		% bone ash
	Initial	Gain		Initial	Gain	
	Diet 44			Diet 44 A (+ fat)		
V, ♂	60	21	44.3	59	0	42.3
VI, ♂	62	22	48.8	62	4	45.0
VII, ♂	61	23	43.9	59	11	42.0
VIII, ♀	59	19	49.3	60	16	44.8
Mean	60.5	21.3	46.6	60.0	7.8	43.5

6. Metabolic study of the effects of fat on the assimilation of Ca and P from a high Ca-low P rachitogenic diet

This experiment was carried out in an attempt to ascertain the mode of action of fat.

(a) *Diets*. As in Exp. 1 B the Gridgeman *et al.* [1939] diet was used as a basis. We have found that this diet is very satisfactory as it consistently produces very severe rickets. Diet 127 A was the same as diet 127 of that experiment, but a different batch of refined semi-hardened ground-nut oil was used (I.V. 75.6, M.P. 28–31°). Diet 144 was similar to diet 126 but maize starch was used instead of rice starch and the non-saponifiable residue of ground-nut oil prepared as described on p. 449 was incorporated at a level equivalent to 20 g. of oil per 132 g. of the diet. This was done to counter-balance any vitamin D which might possibly have been present in the natural oil. The analysis of the diets is given in Table 1.

(b) *Rats and method of feeding*. Seven pairs of littermate male rats were used. Allocation to diets was by toss of coin. The general arrangement of the experiment was as described by Henry & Kon [1937]. The diets were fed mixed with distilled water, 127 A with its own weight and 144 with $1\frac{1}{2}$ times its weight. Calorie intake was equalized between pairs. Distilled water was given to drink and 4 i.u. carotene were administered daily. It was planned to have a 4-week collection period, but the rats scattered their food

Table 9. *Exp. 6. Metabolic data for rats receiving the rachitogenic diet of Gridgeman et al. [1939] with added fat or starch. Diets fed mixed with water*

Rat no.	Wt. of rat (g.)			Diet intake g.	Ca intake g.	Ca excretion (g.)			% Ca retention	P intake g.	P excretion (g.)			% P retention	% bone ash
	Initial	Start of collection				Urine	Faeces				Urine	Faeces			
		period	Final				Diet 127 A (+ fat)	Urine				Faeces	Urine		
1	59	80	101	146.95	1.4823	0.2121	1.2411	+ 0.62	0.3010	0.0017	0.2831	+ 5.38	31.8		
3	58	79	106	162.96	1.6216	0.2834	1.3260	+ 0.75	0.3337	0.0018	0.2968	+ 10.52	29.5		
5	58	78	101	168.47	1.6764	0.2921	1.1708	+ 12.74	0.3450	0.0018	0.2726	+ 20.46	27.9		
7	58	69	94	157.26	1.5649	0.2537	1.3705	- 3.79	0.3221	0.0022	0.2947	+ 7.82	30.0		
9	57	70	93	162.96	1.6216	0.2541	1.3124	+ 3.40	0.3337	0.0016	0.3149	+ 5.15	30.2		
11	55	71	96	161.91	1.6112	0.2899	1.3284	- 0.44	0.3316	0.0018	0.3139	+ 4.80	29.7		
13	58	71	103	177.91	1.7704	0.2780	1.4859	+ 0.37	0.3644	0.0018	0.3380	+ 6.75	28.6		
Mean	57.6	74.0	99.1	162.63	1.6183	0.2662	1.3193	+ 1.95	0.3331	0.0018	0.3020	+ 8.70	29.7		
Diet 144 (+ starch)															
2	60	79	99	192.42	1.4453	0.2834	1.2113	- 3.42	0.3552	0.0013	0.3653	- 3.21	25.0		
4	58	76	93	207.25	1.5567	0.2998	1.3161	- 3.80	0.3826	0.0042	0.3919	- 3.53	25.5		
6	58	74	107	222.12	1.6683	0.3578	1.5920	- 16.87	0.4100	0.0018	0.4551	- 11.44	27.6		
8	58	65	92	204.75	1.5379	0.2829	1.3310	- 4.94	0.3780	0.0029	0.3789	- 1.01	26.4		
10	58	69	90	213.88	1.6065	0.3053	1.3871	- 5.35	0.3948	0.0019	0.3991	- 1.57	24.1		
12	57	68	95	213.40	1.6028	0.3597	1.3118	- 4.29	0.3939	0.0021	0.3866	+ 1.32	28.9		
14	60	79	109	232.20	1.7441	0.3451	1.4192	- 1.16	0.4286	0.0020	0.4099	+ 3.90	26.8		
Mean	58.7	72.9	97.8	212.29	1.5945	0.3191	1.3669	- 5.69	0.3919	0.0023	0.3981	- 2.22	26.3		

badly at the beginning; the start of the balance experiment was therefore postponed for a week and the collection was over 3 weeks only.

(c) *Results.* The results are given in Tables 3 and 9. They confirm in the first instance the findings reported on p. 448. The ash content of the bones on the fat diet was again significantly better than on the starch diet. Both groups of rats developed severe rickets and it will be seen that the rats receiving the starch diet were actually in negative Ca and P balance. The mean losses were about 30 mg. of Ca and 3 mg. of P per rat per week. Brown & Shohl [1930] obtained positive balances with rats receiving the Steenbock 2965 diet; the degree of rickets was, however, much less than in our experiments. With the inclusion of fat in the diet the balances became slightly positive, each rat now retaining about 11 mg. of Ca and 10 mg. of P a week. All seven rats getting the fat retained more or lost less Ca and P than their littermates on the starch diet. Statistical significance attaches, however, only to the second comparison, because of greater variation from rat to rat in the Ca balance. This change in mineral balance was brought about by a significant decrease in the urinary excretion of Ca by all rats receiving fat. The mean faecal excretion was also less, but this was almost entirely due to a relatively large difference within one pair of rats. The urinary excretion of P, in any case very small, was not altered, but the feeding of fat caused a uniform decrease in faecal P.

In view of the lack of agreement as to the effects of vitamin D on Ca and P metabolism of rats receiving high Ca-low P diets it is difficult to say whether the action of fat noticed by us is similar to or different from that of vitamin D itself.

DISCUSSION

From all these different experiments there emerges a well founded observation that vitamin D-free fat exerts a definite beneficial effect on the calcification of rats receiving high Ca-low P rachitogenic diets. This fat effect does not however, take place when the balance of the bone-forming minerals is reversed, that is when the P in the diet is high and Ca relatively low. In this respect the action of fat is qualitatively different from that of vitamin D, which acts whatever the Ca : P ratio of the diet [Shipley, Park, McCollum & Simmonds, 1921].

Our metabolic experiment showed that fat lowered the faecal loss of P and the urinary excretion of Ca. This would be compatible with the conception of Kay & Guyatt [1933] that rickets is a P deficiency disease, at any rate as applied to high Ca-low P rickets in rats. If fat improved the uptake of P from the gut, a certain amount of Ca absorbed but not retained for lack of available P would be combined and hence the urinary Ca would fall. What the actual mechanism of the fat effect is can hardly be deduced from our work. Zucker & Barnett [1922-3] ascribed it to the formation of insoluble Ca salts. The findings of Boyd, Crum & Lyman [1932] and of Hagnell-Zethræus, Neymark & Widmark [1939] speak definitely against this hypothesis. The existence of the fat effect is firmly established by our experiments but further work will be necessary to explain its mechanism.

SUMMARY

1. Several independent experiments have shown that vegetable fats devoid of vitamin D and triglycerides resynthesized from the fatty acids of butter or arachis oil from which the non-saponifiable residue had been carefully removed exerted a definite antirachitic effect on rats receiving high Ca-low P rachitogenic diets, as compared with littermate rats receiving similar diets in which the fat was isocalorically replaced by starch.

2. A similar effect was observed when a synthetic high Ca-low P diet, containing 25 % arachis oil in which all the P was present as inorganic phosphate was compared with a similar diet containing only 5 % of the oil but supplying in equicaloric amounts the same quantities of Ca and P.

3. The calcifying effect of the resynthesized triglycerides of lard and of coconut, olive, linseed and soya bean oils was also demonstrated.
4. Fat exerted no calcifying effect when added to high P-low Ca diets.
5. A metabolic experiment showed that the addition of fat as compared with an equicaloric addition of starch lowers the faecal excretion of P and the urinary excretion of Ca.
6. It is concluded that the fat effect is qualitatively different from that of vitamin D but that it requires further elucidation.

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