creased was studied; pH 5 for phosphate solutions, and pH 5.9 for phosphate-phthalate solutions, were selected as convenient acidities at which to work. With both solutions, the velocity coefficient, k increases as the concentration of the solution is increased. In the phosphate solutions, the rate of aneurin destruction rises, at critical concentrations, through a series of three levels as the concentration is increased from 0.02 to 0.2M (Fig. 3). On the other hand, in the case of the phosphate-phthalate buffer, k increases continually with the concentration over the range studied (Fig. 4).

DISCUSSION

It is apparent that the behaviour of aneurin in the presence of phosphate is varied by the citrate and phthalate ions. Citrate appears to suppress the effect of concentration altogether, and phthalate has altered the relationship between concentration and reaction velocity. This serves still further to emphasize the influence of the various buffer ions on the thermal destruction of aneurin.

The concentration effect has also been found in solutions of yeast extract in which a sudden rise in reaction velocity from one level to another at an almost critical concentration was observed. The concentration/ k curve was very similar to that

found with the phosphate buffer solutions, although it consisted of only one 'step' and the values of k were of a different order from those obtained with the last-mentioned buffer at comparable concentrations.

SUMMARY

1. The effect of the concentration of the buffer salts on the rate of destruction of aneurin was studied in three different buffer solutions between the concentrations 0.2–0.02M and the pH values 3.9–7.0.

2. In phosphate buffer solutions the velocity coefficient, k , is dependent on the concentration of the buffer salts below pH 6. This effect appears to be eliminated by citrate in the phosphate-citric acid buffer solutions and magnified by phthalate in phosphate-phthalate buffer solutions.

3. In phosphate buffer solutions at pH 5, the velocity coefficient rises sharply at critical concentrations of about M/39 and M/19 through three levels when the concentration is increased from 0.02 to 0.2M. However, in phosphate-phthalate buffer solutions at pH 5.9, the relation between the velocity coefficient and concentration changes continuously over the same concentration range:

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REFERENCES

- Booth, R. G. (1943). *Biochem. J.* **37**, 518.
Clark, W. M. (1928). *The Determination of Hydrogen Ions*, 3rd ed. p. 194. London: Baillière, Tindall and Cox.
Farrer, K. T. H. (1941). *Aust. Chem. Inst. J. Proc.* **8**, 113.
Farrer, K. T. H. (1945a). *Biochem. J.* **39**, 128.
Farrer, K. T. H. (1945b). *Biochem. J.* **39**, 261.
Farrer, K. T. H. (1947). *Biochem. J.* **41**, 162.

The Effect of Age and of the Supply of Phosphorus on the Assimilation of Calcium by the Rat*

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Young growing rats retain Ca with almost 100% efficiency from diets suboptimal in this element but adequate in P (Ellis & Mitchell, 1933; Fairbanks & Mitchell, 1936; Henry & Kon, 1939). The last named authors found that under most favourable conditions the daily loss of Ca by a young rat could be as low as 0.3 mg. but that the daily excretion of P could not be brought below 1.3 mg.

* Read in part before the Biochemical Society (Henry & Kon, 1945).

In those experiments the diets contained sub-optimal but appreciable quantities of Ca and one aim of the present study was to find whether with much lower intakes this element would be held with equal tenacity, and to determine the amount of P needed to promote its most efficient utilization.

For this purpose the P content of a diet originally very low in Ca and P (diet 191) was raised by addition of Na₂HPO₄·12H₂O to a level sufficient to cover the minimum unavoidable loss of this element

and to deposit as bone all the Ca present, but insufficient to cover fully the requirements of the soft tissues (diet 192). By doubling this amount of total P, a diet (diet 199) was obtained, in which P was in no way a limiting factor, while by halving it, diet 198 was produced containing only about enough P to balance the unavoidable loss. The retention of Ca and P from these diets was compared with that from the basal diet and also from a diet (diet 193) containing a higher level of Ca and P derived from milk and from sodium phosphate, which had been shown previously to promote an optimal retention of Ca (Henry & Kon, 1939).

About 99 % of the body Ca is in the bones and, as the animal normally matures, a gradual decrease in the retention of this element can reasonably be expected to accompany the progressive mineralization of the skeleton. Bloom (1930), for example,

At the completion of the main experiments further tests were done with special diets to investigate peculiarities of metabolism of Ca by old rats.

EXPERIMENTAL

Plan

The experiment was run in two series. The performance of rats receiving diets 191, 192 and 193 was studied in one, that of rats receiving diets 198, 192 and 199 in the other. Diet 192 was common to both and served as a connecting link. Further diets were tested with the 2-year old rats.

Diets

The basal diet low in Ca and P (diet 191) was that used by us previously (Henry & Kon, 1937, 1939), slightly modified. The composition of this diet and of the other diets derived from it is given in Table 1. To minimize

Table 1. *Composition of the 'synthetic' experimental diets*

(Amounts of components in diet are given as parts.)

Component	Diet 191	Diet 198	Diet 192	Diet 199	Diet 193	Diet 233	Diet 232
Egg-white, commercial, heat-coagulated	13.5	13.5	13.5	13.5	12.0	14.0	21.0
Sugar, granulated ground	9.0	9.0	9.0	9.0	8.0	10.0	10.0
Maize starch	64.8	64.8	64.8	64.8	57.7	53.28	48.68
Margarine fat	10.0	10.0	10.0	10.0	9.9	18.0	13.0
Ca- and P-free salts (Henry & Kon, 1937)	2.7	2.7	2.7	2.7	2.4	3.0	3.0
Spray-dried skim milk	—	—	—	—	10.0	—	—
Na ₂ HPO ₄ ·12H ₂ O	—	0.12	0.48	1.11	1.38	1.39	2.94
CaHPO ₄ ·2H ₂ O	—	—	—	—	—	0.33	1.38

found that rats 33 days old retained 93 % of Ca from a diet containing 0.45 % Ca and 0.34 % P, but that rats 63 days old retained only 58 %. Nicolaysen (1943) reported similar observations. In the experiments of Lantz & Smith (1934) the retention of Ca from the milk-wheat diet (diet B) of Sherman & Macleod (1925) fell from 77 % at 1.5 months to 39 % when the rats were 3 months old.

Fairbanks & Mitchell (1936), Rottensten (1938), Nicolaysen (1943) and other workers have shown that for growing animals the efficiency of utilization of Ca depends primarily on the degree of saturation of the body reserves. Hence with adequate diets it will be closely related to the age of the animal during the period of growth. It seems likely, however, that, quite apart from the Ca content of the tissues, the stage of development of the organism exerts a definite effect. A further aim, therefore, of this inquiry has been to study the gradual changes in assimilation of Ca by the rat as the animal progresses from adolescence to senility.

The metabolic tests used in the first part of this study were therefore repeated with the same rats at intervals for 2 years. During the periods of rest the animals received a stock diet supplying liberal amounts of Ca.

scattering all diets were offered mixed with distilled water. The drinking water also was distilled. The following vitamins were given daily in solution separately from the diet: aneurin, 10 µg.; riboflavin, 40 µg.; pyridoxin, 10 µg.; Ca pantothenate, 300 µg.; inositol, 1 mg.; nicotinic acid, 1 mg.; choline hydrochloride, 3 mg.; *p*-aminobenzoic acid, 3 mg., and cod-liver oil, 2 drops.

The stock diet, given during the rest intervals was as described by Folley, Ikin, Kon & Watson (1938). With it tap water was given to drink. Records of consumption of this dry diet, fresh milk and liver have allowed us to calculate the mean composition of the food eaten by stock colony rats. This is given in Table 2. The Ca content would be augmented by that of the tap water which in this locality amounts to 0.0425 g. Ca/l.

Table 2. *Composition of diet 228*

	(%)
Stock diet	70.7
Dried full-cream milk	26.5*
Dried liver	2.8†

* Equivalent to 208 ml. liquid milk.

† Equivalent to 10 g. fresh liver.

The following additional diets were given to the old rats: (1) the stock colony diet. To simplify feeding, sampling and analysis, fresh milk was replaced by whole dried milk and the liver was steamed, minced, dried, ground and mixed

with the basal diet and milk. This was diet 228. (2) Diet 232, a 'synthetic' type diet, similar in fat, protein, Ca and P content to diet 228. (3) A dried mixed human diet (Macrae, Henry & Kon, 1943). Its Ca and P content was very similar to that of diet 193. (4) Diet 233, a 'synthetic' diet similar to the mixed human diet.

With all these diets the rats were given distilled water to drink; the 'synthetic' diets were supplemented with vitamins as already described. Their compositions are given in Tables 1 and 2 and their analyses in Table 3.

Table 3. *Analyses of the experimental diets**

Diet no.	Ca (%)	P (%)	Ca : P ratio	Moisture (%)
191	0.0114	0.0180	0.63	10.34
198	0.0114	0.0290	0.39	10.07
192	0.0117	0.0592	0.20	10.45
199	0.0113	0.1131	0.10	10.58
193	0.1290	0.2291	0.56	10.84
Mixed human	0.1194	0.2207	0.54	8.74
233	0.1204	0.2190	0.55	7.72
228	0.4610	0.5926	0.78	9.95
232	0.4607	0.5684	0.81	9.30

* A fresh batch of each diet was made and analyzed for each experiment. There were small variations from batch to batch. The figures quoted here are the means for all batches.

Animals

In each series of tests four groups of three, and one group of six, littermate male rats of our own breeding (Folley *et al.* 1938) were used. At the start they were 24–27 days old in the first series and 26–29 days in the second. The initial weights were respectively 37–43 and 43–51 g.

Technique

Littermates were chosen by lot to receive the different diets which were offered in individual metabolic cages. The excreta voided during the first 7 days of any test were discarded and the collection periods lasted 24 days in the first series of experiments and 21 in the second. Metabolic tests were done at the beginning and again when the rats were about 6 months, 1 year and 2 years old. In the first test, six rats were given each diet but in the remaining tests only four animals were used per diet as not more than twelve large metabolic cages of the Hopkins type (Ackroyd & Hopkins, 1916) suitable for adult rats were available at a time. The spare rats were kept as possible substitutes. Several rats died later in the experiment and it proved impossible to follow the original intention of using throughout the same groups of littermates. The error thus introduced was overshadowed by the large differences between the effects of various treatments. During the periods of rest when the rats received the stock diet they were housed four together in ordinary cages. The metabolic cages and the methods of collecting and analyzing the excreta have been described in an earlier publication (Henry & Kon, 1937).

RESULTS

The mean results of the experiment are given in Table 4. It may be convenient to review them under separate headings.

A. *The metabolism of Ca and P in the period of vigorous growth*

Calcium. With diet 191 very low in Ca and P the young rats lost Ca from their bodies at a rate of 3.4 mg. daily, most of it in the urine, the faecal excretion amounting to only 0.1 mg. which is as little as under conditions for optimal retention, e.g. with diet 193 (cf. Henry & Kon, 1939). The urinary loss of Ca diminished with increasing concentration of P in the diet, until for diet 199, which supplied nearly seven times as much P as diet 191, it was only 0.3 mg. daily, rather less than with diet 193 and little more than the minimum observed by us under best possible conditions (Henry & Kon, 1939). It is noteworthy that the faecal excretion was not affected by these changes in intake of P. Diet 192 which contained half as much P as diet 199 apparently supplied just enough of the element to prevent loss of Ca from the body. In fact with the batch of diet used in the first series of experiments there was a small negative balance, while in the second series the diet proved slightly richer in P and the balance became positive.

Phosphorus. The daily loss of phosphorus with the diet deficient in Ca and P (diet 191) was as low as that observed by us previously under conditions presumably conducive to optimal retention (Henry & Kon, 1939). Again the urine contained only 0.1 mg. P daily while ten times this amount, 1.2 mg., was found in the faeces. So little P was present in the diet that, despite the smallness of the excretion, the rats were in negative balance, losing about 0.2 mg. P daily from their bodies. With all other diets the balance was positive, the amount retained daily rising steadily from 1.0 mg. from diet 198, containing twice as much P as diet 191, to over 11 mg. daily from diet 193 with a P content over ten times higher.

B. *Changes in the metabolism of Ca and P with progressing age of the rat*

Calcium. As would be expected, the retention of Ca became less favourable as the rats grew older. Where, from the start, the balance was negative, as with the low Ca and P diet, with diet 198 and, in one series of experiments, with diet 192, the magnitude of the loss increased steadily. For diet 191, for example, the daily loss of 2.7 mg. at 4 weeks rose to nearly 5 mg. at 1 year and to nearly 6 mg. at 2 years.

With two other diets low in Ca but with added P, diet 192 used in the second series and diet 199, the balance was at first positive. It will, in fact, be recalled that from the latter diet the young rats retained Ca with optimal efficiency. In both instances the balance later became negative and

Table 4. Mean values for the Ca and P metabolism of rats, receiving, at different ages, diets of varying Ca and P content

(All values, unless otherwise stated, given on a daily basis.)

(All values, unless otherwise stated, given on a daily basis.)																
Rats used	Age at start of test (weeks)	Length of test (days)	Wt. of rat at start of test (g.)	Gain (g.)	Diet intake* (g.)	Ca		Ca excreted (mg.)		P intake (mg.)	Ca retained		P excreted (mg.)		P retained	
						intake (mg.)	(%)	Urine*	Faeces*		(mg.)	(%)	Urine*	Faeces*	(mg.)	(%)
Diet 191†, first series																
4	5	24	64.8	0.71	6.57 ± 0.16	0.78	3.31 ± 0.26	0.11 ± 0.01	2.46	-344.8 ± 28.6	1.15	0.12 ± 0.004	1.21 ± 0.10	-	0.18	-16.8 ± 10.8
26	4	24	279.7	0.43	17.21 ± 0.43	2.08	1.78 ± 0.24	2.75 ± 0.12	2.45	-118.5 ± 10.5	3.51	0.56 ± 0.069	3.73 ± 0.12	-	0.78	-22.7 ± 6.5
56	4	24	370.5	0.28	17.95 ± 0.30	1.92	3.17 ± 0.56	3.67 ± 0.51	4.92	-256.2 ± 10.7	3.11	0.41 ± 0.064	4.22 ± 0.30	-	1.52	-48.9 ± 10.1
93	3	24	318.3	-0.76	13.48 ± 1.66	1.48	2.66 ± 0.41	4.75 ± 0.35	5.93	-409.3 ± 46.2	2.27	1.46 ± 0.670	4.66 ± 0.27	-	3.86	-185.4 ± 61.5
Diet 198†, second series																
4	6	21	85.7	1.25	9.56 ± 0.39	1.15	3.11 ± 0.24	0.26 ± 0.02	2.22	-193.1 ± 15.7	3.07	0.31 ± 0.16	1.80 ± 0.10	+ 0.96	+ 29.9 ± 7.5	
26	3	21	276.7	0.87	14.90 ± 0.52	1.60	1.56 ± 0.50	2.08 ± 0.45	2.04	-128.8 ± 26.1	4.23	0.59 ± 0.26	3.40 ± 0.28	+ 0.24	+ 4.6 ± 15.9	
55	4	21	383.5	-0.21	17.92 ± 0.73	1.95	1.25 ± 0.08	4.90 ± 0.16	4.20	-217.3 ± 18.3	5.08	2.45 ± 0.16	5.15 ± 0.17	- 2.52	- 51.0 ± 10.2	
93	4	21	384.8	-0.92	17.21 ± 1.84	2.07	1.19 ± 0.05	4.65 ± 0.20	3.77	-191.9 ± 30.5	4.71	2.10 ± 0.67	5.38 ± 0.26	- 2.77	- 65.7 ± 26.4	
Diet 192†, first series																
4	5	24	69.8	1.52	7.72 ± 0.20	0.99	1.10 ± 0.12	0.09 ± 0.01	0.20	-19.8 ± 10.0	4.44	0.53 ± 0.13	1.46 ± 0.08	+ 2.45	+ 54.9 ± 3.7	
26	4	24	284.5	0.60	17.16 ± 1.09	2.06	1.07 ± 0.10	3.61 ± 0.32	2.62	-121.0 ± 21.7	10.54	7.61 ± 0.57	4.13 ± 0.10	- 1.20	- 11.7 ± 2.0	
56	4	24	397.0	0.80	19.25 ± 0.41	2.33	1.21 ± 0.05	5.01 ± 0.51	3.89	-168.5 ± 28.8	11.51	8.42 ± 0.28	4.87 ± 0.29	- 1.78	- 15.7 ± 5.8	
93	4	24	410.0	-0.04	17.13 ± 0.59	1.90	1.20 ± 0.09	5.33 ± 0.14	4.63	-244.2 ± 13.7	9.73	7.13 ± 0.31	5.10 ± 0.12	- 2.50	- 25.8 ± 2.2	
Diet 192†, second series																
4	6	21	85.5	1.87	9.73 ± 0.29	1.16	0.53 ± 0.13	0.16 ± 0.01	0.47	+ 41.1 ± 11.2	5.93	0.69 ± 0.13	1.70 ± 0.06	+ 3.54	+ 59.5 ± 2.7	
26	3	21	306.0	1.05	16.66 ± 0.14	1.81	0.87 ± 0.06	2.41 ± 0.39	1.47	-80.8 ± 23.6	9.80	5.80 ± 0.16	3.82 ± 0.03	+ 0.18	+ 1.6 ± 1.4	
55	4	21	394.2	0.02	16.97 ± 0.94	1.81	1.20 ± 0.06	4.92 ± 0.45	4.31	-244.7 ± 43.9	9.89	7.57 ± 0.35	5.23 ± 0.17	- 2.91	- 31.3 ± 12.0	
93	4	21	422.0	-0.19	18.54 ± 0.51	2.22	1.19 ± 0.21	5.37 ± 0.22	4.34	-196.0 ± 18.8	10.98	7.67 ± 0.52	5.71 ± 0.22	- 2.40	- 22.0 ± 2.6	
Diet 199†, second series																
4	6	21	88.0	1.90	10.11 ± 0.34	1.19	0.29 ± 0.02	0.15 ± 0.01	0.75	+ 63.2 ± 0.8	11.83	5.80 ± 0.18	1.94 ± 0.12	+ 4.09	+ 34.5 ± 1.1	
26	3	21	305.3	0.86	15.24 ± 1.39	1.62	0.77 ± 0.02	2.20 ± 0.31	1.35	-83.5 ± 9.1	16.54	13.32 ± 1.33	3.37 ± 0.17	- 0.15	- 1.1 ± 0.9	
55	4	21	436.2	-0.19	18.65 ± 1.27	2.05	1.20 ± 0.08	5.19 ± 0.24	4.34	-219.2 ± 26.1	20.94	17.46 ± 1.84	5.03 ± 0.23	- 1.55	- 7.9 ± 2.4	
93	4	21	394.3	-1.32	17.80 ± 1.66	2.12	1.12 ± 0.14	5.09 ± 0.20	4.09	-201.2 ± 30.5	20.39	16.72 ± 1.11	5.18 ± 0.19	- 1.51	- 9.1 ± 6.7	
Diet 193†, first series																
4	5	24	73.6	3.28	10.24 ± 0.49	12.99	0.35 ± 0.04	0.11 ± 0.02	12.53	+ 96.4 ± 0.6	23.18	10.12 ± 0.44	1.77 ± 0.12	+ 11.29	+ 48.6 ± 0.50	
26	4	24	351.3	0.67	18.96 ± 0.58	24.29	2.86 ± 0.10	16.03 ± 0.31	5.40	+ 22.1 ± 1.8	44.97	32.88 ± 1.12	9.00 ± 0.33	+ 3.09	+ 6.8 ± 0.83	
56	4	24	455.2	0.70	20.79 ± 1.17	26.91	3.30 ± 0.21	20.90 ± 0.86	2.71	+ 9.9 ± 1.7	47.54	32.75 ± 1.77	11.78 ± 0.57	+ 3.01	+ 6.2 ± 0.69	
93	3	24	409.7	-0.04	18.82 ± 0.95	24.78	4.80 ± 1.54	21.63 ± 0.45	1.65	- 6.7 ± 1.4	42.17	31.09 ± 2.74	13.91 ± 0.77	- 2.83	- 5.1 ± 2.84	
Diet 228†, first series																
102	8	14	364.1	-0.44	16.05 ± 0.54	74.27	3.86 ± 0.58	69.64 ± 1.94	0.77	+ 0.96 ± 0.6	96.67	40.13 ± 1.53	53.21 ± 1.38	+ 3.33	+ 3.5 ± 1.7	
Diet 228†, second series																
108	5	14	363.6	-0.63	14.60 ± 1.11	67.09	3.67 ± 0.97	61.14 ± 4.23	2.28	+ 3.4 ± 1.3	85.12	37.25 ± 4.11	46.69 ± 2.73	+ 1.18	+ 1.4 ± 1.0	
Diet 232†, second series																
108	5	14	343.4	-0.57	13.56 ± 1.33	62.45	2.42 ± 0.32	58.99 ± 6.43	1.04	+ 2.1 ± 2.0	77.04	39.46 ± 4.46	36.82 ± 4.52	+ 0.76	+ 1.7 ± 1.9	
Mixed human diet†, second series																
113	4	14	348.2	-1.73	11.67 ± 1.30	13.93	1.81 ± 0.56	14.26 ± 1.74	2.14	- 14.9 ± 5.0	25.76	18.23 ± 2.72	10.53 ± 0.54	- 3.00	- 12.1 ± 3.6	
115	4	14	324.0	-0.74	12.63 ± 1.76	15.07	2.72 ± 0.58	14.85 ± 1.72	2.50	- 19.4 ± 8.2	27.86	18.37 ± 3.18	8.46 ± 1.46	+ 1.03	+ 3.1 ± 3.0	
Diet 233†, second series																
113	3	14	353.7	-1.29	12.52 ± 0.73	15.08	1.85 ± 0.37	15.36 ± 0.46	2.13	- 14.4 ± 3.9	27.43	20.56 ± 0.73	9.36 ± 0.41	- 2.49	- 9.6 ± 6.3	
115	3	14	335.7	-1.93	12.09 ± 0.37	14.55	2.74 ± 0.44	13.07 ± 0.73	1.26	- 6.9 ± 8.5	26.47	19.16 ± 1.57	8.34 ± 0.42	- 1.03	- 3.7 ± 5.5	
* Values quoted with their standard error of the mean.																
† Details of the diets are given in Tables 1, 2 and 3.																

* Values quoted with their standard error of the mean. † Details of the diets are given in Tables 1, 2 and 3.

deteriorated as the rats grew older. For example, the daily retention from diet 199 of 0.8 mg. Ca by 4 weeks old rats changed to a loss of 1.4 mg. at 6 months and of over 4 mg. at 1 and 2 years of age. It is of interest that, while the retention of Ca by young rats from the low Ca diet was markedly affected by the P content of the diet, rats over a year old lost about 4 mg. daily whether the diet contained 0.03 or 0.11 % P, though those receiving the diet with only 0.02 % P lost 5-6 mg.

The most striking effect of age on the assimilation of Ca was undoubtedly that observed with diet 193, suboptimal in Ca, but much more adequate in this respect than any of the other diets, and with ample phosphorus. From the almost quantitative retention, amounting to 96 % at the stage of rapid growth, the assimilation decreased steadily, as would be expected, until at 1 year old the rats were almost in Ca equilibrium, retaining only some 3 mg. daily, or 10 % of the intake. We had expected that they would thereafter remain in Ca balance. In fact, however, a year later the rats were losing Ca from their bodies at the rate of nearly 2 mg. a day while receiving the same diet from which they had retained it so efficiently in their early youth.

Further tests were done with these rats to find out whether the negative balance was an unavoidable effect of senility or if it could be prevented by the use of a diet richer in Ca and P. The latter proved true with the modified stock diet (diet 228) which maintained the rats in Ca equilibrium.

As this diet is composed of natural ingredients, the question arose whether a 'synthetic' diet (diet 232) would do equally well. Parallel tests were therefore done with littermates in the second series of experiments. These showed no difference between the two diets (228 and 232), thus indicating that the maintenance of Ca equilibrium in old age is primarily a question of the supply of Ca and P. This view was strengthened when it was found in tests which followed that a reversion to a diet of lower Ca content led to a loss of Ca from the body whether the diet was 'natural' or 'synthetic'. The 'natural' diet was the dried human diet described by Macrae *et al.* (1943) and the 'synthetic' ration (diet 233) was similar to it in fat, protein, Ca and P content.

In discussing with us the results of our experiments, Dr D. P. Cuthbertson suggested that the negative Ca balance observed with old rats receiving diet 193 might have been due to their taking longer than when young to reach equilibrium after a change in diet, and that it was possible that the 7 days' preliminary period after the switch-over from the stock diet given during the rest period to diet 193 was, for them, too short. If this were so, a prolongation of the experimental period should restore Ca balance. To test this point the comparison of the 'natural' and the 'synthetic' rations was continued

for another 2 weeks directly on completion of the tests described above. Table 4 shows that the loss of Ca persisted unchanged with both diets. It seems therefore that the inefficient retention of Ca by old rats is not due to delayed acclimatization but is inherently connected with senescence. In all cases the deterioration was largely due to less efficient uptake from the gut. The old rats consistently lost weight during all the metabolic tests; whatever the effect of this loss on the retention of minerals it applied equally to all results.

Phosphorus. The retention of P followed generally the Ca trends. The percentage retention from all diets decreased as the rats grew older with the completion of growth of skeleton and active tissue. The increased loss was mostly in the urine especially with the diets containing the larger amounts of P derived from sodium acid phosphate.

DISCUSSION

A. *The metabolism of Ca and P in the period of vigorous growth*

Calcium. It may be stressing the obvious to cite our experiments as a demonstration of the intimate metabolic interrelation of Ca and P. We do this without apology as this truism has often been glaring enough to be overlooked. The influence of the one mineral on the utilization of the other is indeed striking as shown so clearly now by the effect of an increasing supply of P on the retention of Ca by the young growing rat. In fact, while with a diet containing only 0.01 % Ca and 0.02 % P there was a heavy drain of Ca from the body, an increase of the P content to 0.1 % led to a positive balance of Ca despite the minuteness of the supply. We had previously shown in agreement with other workers (Henry & Kon, 1939; Ellis & Mitchell, 1933) that with suboptimal but substantial supplies of Ca the retention may be so good that only unavoidable traces are lost from the body. The present findings show that this is equally true when the diet is much poorer in Ca, provided other conditions are conducive to maximum retention. For the period of vigorous growth in the rat we cannot therefore confirm the view expressed by Smith & Smith (1934) that 'there is a constant metabolic demand—a "wear and tear" requirement—for calcium operative even when there exists an absolute lack in the diet'. This ability to retain all but traces of Ca may only last a short time. Our rats were already at 6 months excreting so much more Ca that they were in negative balance with the low Ca diet just mentioned. The rats of Smith & Smith (1934) were about 4 months old and it may well be that their findings would have been similar to ours had their rats been younger.

Phosphorus. For the growing animal P is in a sense even more essential than Ca as the former is needed in more nearly equal proportions for the formation of bone and of extra-skeletal tissues, while 99% of the Ca is concentrated in the bones. We have previously shown (Henry & Kon, 1937, 1939) that when the supply of both minerals is inadequate the soft tissues take precedence over the bones in respect of P. At 4 weeks of age the rats receiving the various diets were all growing though at rates increasing with the P intake. Those receiving diet 191 were actually losing P from their bodies, the others were retaining roughly from 1 to 4 mg. P/g. gain in weight. For the calculation of the amount of P available for the formation of the soft tissues the amounts retained in, or liberated from, the bones must be taken into account. This we have done by assuming that 99% of Ca is in the bones, that the Ca : P ratio of bone is 2.3 : 1 (Brooke, Smith & Smith, 1934; Kramer & Shear, 1928), and that the bone minerals are liberated in that ratio in negative Ca balance, and deposited in positive. Values so obtained combined with the P balance give the P available for extra-skeletal use. Table 5 shows the

The large excess of P necessary to promote the optimal retention of Ca is worthy of note. For diet 199, with which these optimal conditions were apparently reached, the P : Ca ratio was 10 : 1. This marked increase in P with the Ca remaining reasonably constant did not affect the faecal excretion of the latter but brought down the urinary loss from 3.31 (diet 191) to 0.29 mg./day (diet 199). If bones had precedence over the soft tissues in the competition for P, optimal retention of Ca could presumably be achieved with an amount of P sufficient to retain as bone the Ca otherwise excreted in the urine with diet 191. The additional quantity required for this purpose can be calculated as

$$\frac{3.31 - 0.29}{2.3} = 1.3 \text{ mg. P/day.}$$

As diet 191 supplied 1.15 mg. P/day, the total needed would amount to 2.45 mg. P/day. In fact 11.8 mg. proved necessary, and the intermediate amounts of 3.1 mg. supplied by diet 198 and 4.5–6.0 mg. supplied by diet 192 were insufficient to prevent a marked loss of Ca in the urine. It seems evident that only a fraction of the P was available under these conditions for binding Ca in the bone, or that, in other words the demands of the soft tissues were 'setting the pace'.

The calculations of P available for soft tissues were based on the assumption that in negative Ca balance the Ca is derived from the bone minerals as a whole with a corresponding release of the P. Brooke *et al.* (1934) have shown, however, that, under certain conditions, the Ca is preferentially withdrawn from the bone carbonate. Their diet contained rather more P than our diet 199 with which the Ca balance was positive and the question of P derived from bones did not arise. Whether their findings apply to the diets and conditions used by us is an open question. It might well be argued on teleological grounds that the marked loss of Ca with diets deficient in both Ca and P is part of a mechanism intended to supply P for growth of soft tissues by the dissolution of bone. That this loss of Ca is not unavoidable is shown by its becoming negligible in the presence of sufficient P. A withdrawal of Ca from the carbonate fraction of bone only would seem purposeless under such conditions.

B. Changes in the metabolism of Ca and P with progressing age of the rat

Calcium. In our experiments, the increasing inefficiency of Ca retention is the striking feature of advancing age. It will be recalled that during periods of rest between metabolic tests all the rats received the fully adequate stock diet. The Ca stores of the adult rats were therefore probably saturated at the beginning of each experiment and the degree of

Table 5. Calculated amounts of P available for deposition in the soft tissues of young rats during the period of vigorous growth (4 weeks) and after the cessation of vigorous growth (26 weeks)

Diet no.	4-weeks old rats		26-weeks old rats
	mg. P available/g. gain in wt.	% of normal requirement	mg. P available/g. gain in wt.
191	1.37	52.1	0.70
198	1.54	58.6	1.29
192 (1st series)	1.67	63.8	–0.12
192 (2nd series)	1.87	68.2	0.77
199	1.98	75.7	0.50
193	1.80	68.7	1.15

results of such calculations. The relative constancy of the amount of P deposited in the soft tissues/g. gain in weight is remarkable in view of the wide divergency of the exogenous supply. A similar calculation applied to the values of Sherman & Quinn (1926) for 'normal' rats of the same age gives a retention in the soft tissues of 2.62 mg. P/g. gain in weight. Rats in the present experiment satisfied, therefore, their extra-skeletal requirements to an extent varying from 52.1% for diet 191 to 75.7% for diet 199. More direct earlier measurements based on carcass analysis yielded (Henry & Kon, 1939) a figure of 85% for a diet similar in composition to diet 193, but by the present method of calculation, the only one possible in the absence of carcass analysis, the figure is reduced to 77%. The indirect method yields, therefore, lower figures.

saturation was in all probability the same for rats 1 year old as for those 2 years of age, yet the former managed to maintain Ca equilibrium with diet 193 whereas the latter did not. Increasing katabolism of the skeleton in senescence is the most likely explanation of this difference, and indeed indications are not lacking from animal experiments or from observations on man of the loss of bone salts in old age (McCay, Crowell & Maynard, 1935; Meulengracht, 1938; Todd, 1942; Stare, 1943). In the vigorous anabolic phase of growth, deposition of bone elements is possible even with low concentrations of these elements in the diet. As the skeleton matures, the flow in the direction of the skeleton becomes more sluggish and finally stops. After a period of equilibrium, the tide turns with advancing age but the ebb can be stopped, as our experiments show, by raising the level, and thus increasing the potential, of the bone minerals in the diet. If adequacy of supply can be measured by rate of retention or, later, by maintenance of equilibrium, two peaks of Ca need will be found, one in youth and the other in senility. There is much evidence to show that a good supply of Ca is advantageous for the growing animal (Toepfer & Sherman, 1936; Sherman & Campbell, 1937; Lanford, Campbell & Sherman, 1941; Campbell & Sherman, 1945). It is less certain whether maintenance of Ca equilibrium in advanced age is equally desirable. In senility Ca tends to deposit in tissues other than the bone, and it may be that this is a direct result of the bone atrophy, as in the mobilization of bone salts in intoxication with vitamin D. If that were true the damming of bone katabolism by increased supply of Ca and P in the diet might well prove beneficial. It might on the other hand itself contribute directly to the aberrant deposition of Ca.

Phosphorus. By 6 months the growth of the active soft tissues in the rat is nearly completed and further gain in weight is largely due to deposition of fat. It is therefore not surprising that a calculation of the P available for soft tissues/g. gain in weight (p. 174) should yield figures much more variable than those obtained at the stage of vigorous growth (Table 5). Calculations for rats 1 and 2 years old are meaningless since by then growth had entirely stopped or the rats were actually losing weight.

On the whole the retention of P followed with age the pattern of the retention of Ca. Again, for each diet the balance became increasingly less satisfactory as the animals grew older. An increase in the supply of Ca and P for old rats re-established a positive balance.

SUMMARY

1. The Ca retention by rats from diets containing 0.011 % Ca and 0.018–0.113 % P has been studied at intervals in two series of experiments from the time the rats were 1 month old until they attained the age of 2 years. In one series a simultaneous comparison was made with a diet containing 0.129 % Ca and 0.229 % P (diet 193). Between metabolic tests the animals were maintained on a stock diet containing adequate Ca and P.

2. Young actively growing rats were in negative Ca balance on the diet containing 0.011 % Ca and 0.018 % P. With increasing P content of the diet Ca retention improved and at 0.059 % P the animals were just in balance, while at 0.113 % P they retained 63 % of the ingested Ca. The daily excretion of 0.44 mg. Ca was almost identical with the figure of 0.46 mg. obtained with diet 193, considered from previous experience to induce optimal Ca retention.

3. Poorer Ca absorption, as shown by increased faecal Ca, was observed with all diets as the age of the rats increased from 1 month to 2 years. This was most striking with diet 193 where a positive Ca balance of 96.4 % at 1 month became negative to the extent of 6.7 % at 2 years of age.

4. Two-year old rats could be brought into Ca balance by increasing the dietary Ca and P to 0.461 % and 0.593 % respectively. It was immaterial whether the diet was composed of 'natural' or of 'synthetic' foodstuffs.

5. The retention of P was roughly parallel to that of Ca.

6. The evidence suggests that in young, actively growing rats the soft tissues take precedence over bone in the competition for P.

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REFERENCES

- Ackroyd, H. & Hopkins, F. G. (1916). *Biochem. J.* **10**, 551.
 Bloom, M. A. (1930). *J. biol. Chem.* **89**, 221.
 Brooke, R. O., Smith, A. H. & Smith, P. K. (1934). *J. biol. Chem.* **104**, 141.
 Campbell, H. L. & Sherman, H. C. (1945). *Amer. J. Physiol.* **144**, 717.
 Ellis, M. & Mitchell, H. H. (1933). *Amer. J. Physiol.* **104**, 1.
 Fairbanks, B. W. & Mitchell, H. H. (1936). *J. Nutr.* **11**, 551.
 Folley, S. J., Ikin, E. W., Kon, S. K. & Watson, H. M. S. (1938). *Biochem. J.* **32**, 1988.
 Henry, K. M. & Kon, S. K. (1937). *Milk and Nutrition*, Part 1, p. 9. Reading: Nat. Inst. Res. Dairying.
 Henry, K. M. & Kon, S. K. (1939). *Biochem. J.* **33**, 173.
 Henry, K. M. & Kon, S. K. (1945). *Biochem. J.* **39**, xxi.

- Kramer, B. & Shear, M. J. (1928). *J. biol. Chem.* **79**, 147.
- Lanford, C. S., Campbell, H. L. & Sherman, H. C. (1941). *J. biol. Chem.* **137**, 627.
- Lantz, E. M. & Smith, M. C. (1934). *Amer. J. Physiol.* **109**, 645.
- McCay, C. M., Crowell, M. F. & Maynard, L. A. (1935). *J. Nutrit.* **10**, 63.
- Macrae, T. F., Henry, K. M. & Kon, S. K. (1943). *Biochem. J.* **37**, 225.
- Meulengracht, E. (1938). *Lancet*, **235**, 774.
- Nicolaysen, R. (1943). *Acta physiol. scand.* **5**, 200.
- Rottensten, K. V. (1938). *Biochem. J.* **32**, 1285.
- Sherman, H. C. & Campbell, H. L. (1937). *J. Nutrit.* **14**, 609.
- Sherman, H. C. & Macleod, F. L. (1925). *J. biol. Chem.* **64**, 429.
- Sherman, H. C. & Quinn, E. J. (1926). *J. biol. Chem.* **67**, 667.
- Smith, A. H. & Smith, P. K. (1934). *J. biol. Chem.* **107**, 681.
- Stare, F. J. (1943). *Proceedings Research Conference on the Relation of Nutrition to Public Health*, p. 7. New York: The Nutrition Foundation.
- Toepfer, E. W. & Sherman, H. C. (1936). *J. biol. Chem.* **115**, 685.
- Todd, T. W. (1942). *Problems of Ageing* (ed. E. V. Cowdry), 2nd ed. p. 322. Baltimore: The Williams and Wilkins Co.

Studies on the more Soluble Complexes of Soil Organic Matter

1. A METHOD OF FRACTIONATION

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This paper is intended as the first of a series dealing with the so-called 'fulvic acid' fraction of soil organic matter. Fulvic acid (after Oden, 1919) is that part of soil organic matter which is peptized by alkali but remains in solution on precipitation of the 'humic acids' with acid. It is proposed in this paper to set forth the method of fractionation adopted and in subsequent papers to deal in more detail with the character of the isolated complexes.

The fulvic acid fraction has not up to now received, in comparison with the humic precipitate, the attention it warrants. As it contains the more soluble soil organic constituents, it should be the fraction most intimately associated with plant growth, and thus be of the utmost importance. Moreover, from recent work by Tiurin (1944) it appears to be the fraction of soil organic matter which effects the mobilization of the sesquioxides, during podzolization.

Little work has been done on the actual isolation of the components of this fraction since Schreiner & Shorey (1909, 1910, 1911) isolated several simple degradation products (amino-acids, purine bases, sugars) in small yield. Since that early period, however, the methods of isolation of organic constituents from natural sources have been greatly improved and augmented.

Since the fulvic extract consists of water soluble components it was thought that it might be fractionated by a method using a chromatographic or general adsorptive technique. After tests under varying conditions with activated alumina, charcoal, various grades of fuller's earth and other clay adsorbents, charcoal was selected as the most suit-

able. The charcoal used was of the quality described as 'animal, purified' (Hopkin & Williams, Ltd).

It was found that if the fulvic extract was adjusted to pH 2.5-3.0 and then run through a layer of charcoal of suitable dimensions, over 90% of the organic matter was adsorbed by the charcoal. This organic matter could be subsequently extracted with various solvents and thus separated into distinct fractions. The details of a method were determined and applied to the fulvic extracts of four soils.

EXPERIMENTAL

Materials

The soils used were as diverse as possible and consisted of samples of

- (1) Heather raw humus,
- (2) Gardener's 'turf' soil,
- (3) *Sphagnum*-cottongrass peat,
- (4) Pine raw humus.

Preparation of the fulvic extracts

A weight of the original oven-dried (at 65°) soil containing 100 g. of organic matter was stirred with 10 l. of 0.5N-sodium hydroxide for 48 hr. It was then allowed to settle and the supernatant dark liquor siphoned off. This suspension was extremely difficult to filter by the usual methods. It was found, however, that when the solution was run through a wide cylinder packed with cotton wool to remove the finely suspended material it could be readily filtered under pressure through a sintered glass funnel (pore size 5-10 μ). Filtration of 10 l. by this method took on the average only 24 hr. To the residue a further 10 l. 0.5N-sodium hydroxide was added and the stirring, settling, and siphoning repeated. The residue was then washed by