VITAMIN D AND GROWTH [1]

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FOUR FIGURES

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Improved growth as the result of the administration of vitamin D has been reported by many investigators. For example, there are available reports for the rat, for the turkey (Baird and Greene, '35; Robertson et al., '41), for the pig (McGowan, '33; Senior, '40; Brion et al., '51), for the chick (Bethke et al., '29; McGowan and Emslie, '34; Migicovsky and Emslie, '47; Buckner et al., '52), and for the infant (reviewed by Jeans, '51).

In the numerous reports on the rat the data often are hard to evaluate because of the variability in the amounts of vitamin A, Ca and P in the diets. Improvement in growth usually was accepted casually as a normal reaction to the correction of a dietary deficiency. Steenbock and coworkers ('23, '24a, '24–5b) used the induction of growth-promoting properties in foods by irradiation as evidence of the synthesis of vitamin D. But with some diets, for example those in which the Ca/P ratio was very high it soon became evident that vitamin D did not induce growth (Bethke et al., '23–'24; Bethke et al., '32; Kramer and Howland, '32). Dodds and Cameron ('43) obtained increased growth when vitamin D was given to rats made rachitic on Steenbock and Black's ration

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2965 but not when given as a supplement to the ration from the beginning. Schneider and Steenbock ('39) and Jones ('44) found that vitamin D actually inhibited growth when added to diets very low in P but normal in Ca.

A considerable number of investigators have fed diets in which the ratio of Ca to P was very low. While Fairhall ('28), with diets especially high in P stressed primarily the effect of vitamin D on calcification, inspection of his data reveal a pronounced beneficial effect on growth. Shohl ('36), and Jones and Cohn ('36) fed diets high in P but failed to report their effect on growth. Greenberg and coworkers (see Boelter and Greenberg, '41a,b) fed cod liver oil with all of their diets. Sobel et al. ('45) and Carlsson ('52) found a slight increase in growth with cereal diets which were quite high in P but low in Ca. Morgan et al. ('40), using purified diets, obtained more growth by the addition of vitamin D to low-Ca diets than to low-P diets. Migicovsky and Emslie ('47) and Consuret ('54) also obtained the greatest increase in growth with diets high in P.

The lead to our experimental work was provided by the observation of Bellin et al. ('54) that considerable growth could be produced in the rat, though only for a limited time, by the addition of vitamin D to a diet which contained 0.62% P and as little as 0.03% Ca. Without this addition even maintenance was impossible, and early death supervened. It was furthermore observed, though not reported at the time, that in spite of this growth the cartilaginous metaphyses of the long bones were narrower than in the normal rat and that the addition of vitamin D increased their width slightly.

Our present report deals primarily with observations on the relation of vitamin D to growth with variations in the intake of Ca and P.

EXPERIMENTAL

Approximately 30-day-old male Sprague-Dawley albino rats, weighing 70 to 80 gm, were used in all experiments except in one in which both males and females were used to determine

the effect of sex. They were housed individually in hanging wire-screen cages. Food and distilled water were kept available for their ad libitum consumption.

As evidence of the nutritive state of our rats when started on experiment the following data pertinent to the interpretation of our experiments were obtained on 9 male rats as received in different shipments: weight, 73 gm; serum inorganic P, 10.5 mg %; serum Ca, 10.4 mg %; extracted femur weight 0.1176 gm, ash weight 0.0638 gm, organic weight 0.0539 gm, per cent ash 54.3, length 22.1 mm; radial metaphyseal width 0.22 mm.

The rats were fed a basal diet composed of glucose [2] 67, steamed egg white 18, cottonseed oil [3] 10, roughage [4] 3, Ca-P free salts 2, and a water-soluble vitamin supplement. The composition of the salts and vitamin mixture has been described previously (Steenbock et al., '51). Supplements of calcium or phosphorus were given at the expense of the glucose ingredient except in the experiments which involved dietary increases in Ca or P. In these, they were given as additions to the rations. Calcium was usually supplied as $CaCO_3$, and P as a neutral equimolar mixture of KH_2PO_4 and K_2HPO_4, except when very high levels of Ca were desired. In these instances $CaCO_3$-$Ca_3(PO_4)_2$ mixtures were used to keep the salt content of the diet at low levels. These mixtures were prepared by adding weighed amounts of $CaCO_3$ to standardized H_3PO_4 solutions, evaporating and then desiccating them under infrared lamps, and grinding with a mortar and pestle to pass an 80-mesh sieve. The various diets were prepared and stored without the oil ingredient until used. Following addition of the oil, any unused portions were stored in the refrigerator. All diets were analyzed for Ca and P.

Fat-soluble vitamins A, E, and K were given orally in a cottonseed oil solution three times per week with a medicine dropper. The minimum amounts given to each rat weekly were

[2] Cerelose.
[3] Wesson
[4] Ruffex.

70 μg β-carotene, 875 μg α-tocopherol, and 105 μg 2-methyl-1, 4-naphthoquinone. Vitamin D_2 was given orally, when required, as a cottonseed oil solution of calciferol, using a calibrated one milliliter syringe. When a vitamin D supplement was indicated, each rat was given 75 I.U. of vitamin D_2 on the first day of the experiment and on every third day subsequently.

The usual length of the experimental periods was 21 days. At the completion of an experiment, the rats were usually anesthetized with ether and killed by severing the carotid artery. Blood sera were analyzed for P by the colorimetric method of Fiske and Subbarow ('25) and for Ca according to the Clark and Collip ('25) modification of the Kramer and Tisdall ('21) method. The Ca was precipitated directly as the oxalate, dissolved in 1N H_2SO_4 and titrated with N/100 $KMnO_4$.

Bone ash determinations were made on alcohol-ether extracted femora. The bones were dried at 105°C., and ashed at 1400°F. for 5 hours. Organic bone was assumed to be the difference between the weight of the bone and the weight of its ash.

The distal ends of the ulnae and radii were split, washed, and treated with 1.5% $AgNO_3$. Photographs of the sections were taken for measurement of the cartilaginous metaphyses.[5] Sometimes the femora were measured with vernier calipers as additional evidence for establishing changes in growth, but for the most part we relied upon comparative body weights as a measure of comparative growth as no signs of obesity or unusual body symmetry with changes in weight were noted.

RESULTS

Table 1 brings out several noteworthy facts: that the rats were able to live for many weeks on a diet containing only 0.018% Ca; that growth was strongly inhibited as the

[5] The term metaphysis is used here to designate that area of the long bone which lies between the epiphysis and the calcified diaphysis.

TABLE 1

Effect of varying the P content of a low-Ca diet [1] on growth, blood and bone

P CONTENT	D_2	NO. OF RATS	FOOD INTAKE	GAIN IN WT.	SERUM P	SERUM Ca	FEMUR			
							Dry wt.	Ash wt.	Organic wt.	Ash
%			*gm*	*gm*	*mg %*	*mg %*	*gm*	*mg*	*gm*	%
0.016	yes	6	244	58	7.6	10.9	0.119	30	0.088	25.5
	no	6	207	45	2.8	10.1	0.141	33	0.107	23.6
0.105	yes	6	287	107	9.0	10.6	0.172	65	0.107	37.8
	no	6	174	20	10.2	5.9	0.152	58	0.094	38.2
0.187	yes	6	287	101	8.5	10.6	0.178	70	0.107	39.6
	no	6	160	13	8.9	6.0	0.150	62	0.087	41.7
0.296	yes	12	279	96	9.2	9.9	0.164	67	0.097	40.9
	no	12	155	13	9.9	6.8	0.142	60	0.081	42.6
0.301	yes	3 [2]	248	73	8.5	10.1	0.153	69	0.084	45.0
	no	3 [2]	159	11	9.4	5.2	0.124	60	0.064	48.0
0.304	yes	4 [3]	1036	150	7.2	9.3	0.172	75	0.096	43.9
	no	4 [4]	347	32	7.9	5.2	0.151	61	0.089	40.7
0.573	yes	6	260	93	10.2	11.1	0.143	60	0.083	42.3
	no	6	102	6	11.9	5.8	0.113	52	0.060	46.8
1.202	yes	6	231	82	9.4	9.3	0.166	64	0.102	38.9
	no	6	109	2	11.2	6.5	0.134	59	0.075	43.8
1.168	yes	4 [3]	970	135	8.7	8.2	0.164	70	0.094	42.7
	no	4 [4]	367	2	14.9	5.0	0.123	55	0.067	45.0

[1] All diets contained 0.018% Ca.

[2] Females instead of males.

[3] All rats were continued on experiment for three weeks except some which were killed after 13 weeks.

[4] All rats were continued on experiment for three weeks except some which died after 9 weeks.

amount of P in the diet was increased and that the addition of vitamin D not only prevented this inhibition but stimulated growth greatly. This stimulation was not limited to a three-week period, but continued for 10 weeks after which maintenance supervened for at least three weeks longer (fig. 1). In the absence of vitamin D, death occurred in one-half of the rats after 9 weeks on the ration; frequently after the appearance of tetany, diarrhea, dry, scaly paws and tails, and bloody nares. Our data on growth inhibition resulting

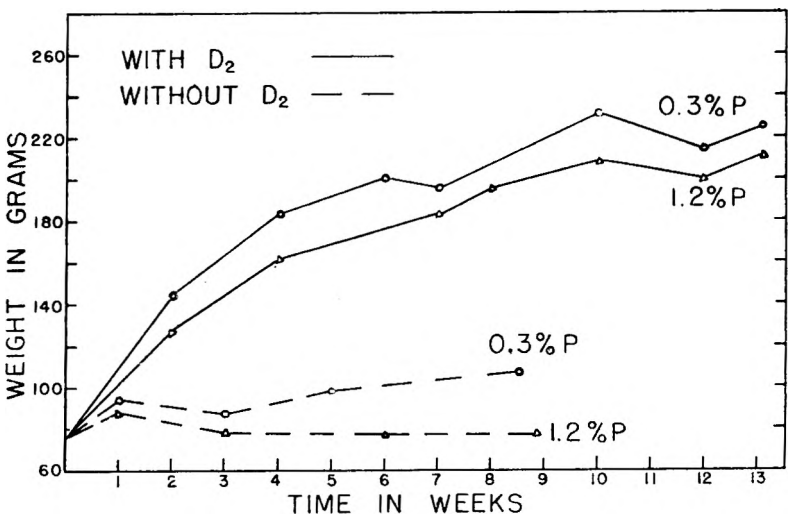

Fig. 1 Growth on high-P, low-Ca diets for an extended period of time.

from an increase of the P content of the diet reaffirmed the earlier findings of Bethke et al. ('23–4, '32) and of Sobel et al. ('45).

It will be noticed (table 1) that the feeding of high-P, low-Ca diets induced a hyperphosphatemia in the absence of vitamin D. This was particularly noticeable in the rats which had been 9 weeks on a diet containing 1.2% P. Sera Ca levels were greatly depressed by the addition of P in all non-D groups but were restored to approximately normal values by vitamin D.

The bone data reveal no outstanding change in the weight of bone ash during the experimental period, even when vitamin D was given. In fact, the percentage of ash was actually decreased by the vitamin, for in harmony with the resultant increase in body growth, the increase in organic bone was large. Concurrently the bones were increased in length, for example, after three weeks on the 0.3% P, low-Ca ration, the length of the femurs was increased from 25.1 to 28.9 mm. After 13 weeks the differential in length was increased still more, though the increase in weight was small. These bones were almost hollow, with only a thin cortex, and were severely congested. In contrast to the soft bones with hypertrophied ends which are produced by high-Ca, low-P rachitogenic diets, the bones produced by these high-P diets were brittle and porotic, without evident enlargement of the ends. The percentage of ash was always reduced by vitamin D with one exception (0.30% P) in which the non-D and D rats were not comparable because the former died earlier.

Another unusual effect of vitamin D with these diets was that it increased the width of the abnormally narrow cartilaginous metaphyses from a width of 0.10 mm to approximately a normal width of about 0.25 mm. So far as we know, this effect of vitamin D is produced only with rats on diets with low Ca/P ratios. Over a period of time longer than three weeks, as growth ceased, the cartilaginous metaphyses were almost obliterated, whether or not vitamin D was supplied.

The dietary lack of both P and Ca elicited slightly different reactions, for while vitamin D was apparently essential at these low levels of intake (0.018% Ca, 0.016% P) for the maintenance of a normal level of serum P, it was not required for the maintenance of a normal level of serum Ca. The bones were very soft, porous and flexible. On the basis of their low ash content and on the result of previous balance studies, presented in part by Bellin et al. ('54), it is suggested that resorption occurred to meet the P requirements of the soft tissues for growth (Day and McCollum, '39).

TABLE 2

Effect of varying dietary Ca content with adequate P intake[1] on growth, blood and bone

Ca CONTENT	Ca/P RATIO	D_2	RATS	FOOD INTAKE	GAIN IN WT.	SERUM P	SERUM Ca	FEMUR			
								Dry wt.	Ash wt.	Organic wt.	Ash
%				*gm*	*gm*	*mg %*	*mg %*	*gm*	*gm*	*gm*	%
0.21	0.68	yes	5	242	97	9.6	10.2	0.213	0.108	0.104	51.0
		no	6	180	32	9.6	8.0	0.159	0 076	0.083	47.8
0.43	1.39	yes	16	230	100	8.6	10.8	0.239	0.131	0.107	54.9
		no	16	171	55	9.0	7.2	0.181	0.104	0.089	50.6
0.45[2]	1.43	yes	4	284	114	9.6	10.3	0.243	0.138	0.105	56.6
		no	4	190	55	9.2	6.4	0.173	0.089	0.084	51.5
0.43[3]	1.37	yes	4	266	107	9.0	9.9	0.244	0.134	0.110	54.8
		no	4	205	54	9.3	5.9	0.179	0.087	0.091	48.8
0.62	1.98	yes	6	288	100	9.3	11.4	0.252	0.139	0.113	55.1
		no	5	214	59	12.1	7.8	0.197	0.095	0.101	48.3
0.83[2]	2.66	yes	6	279	101	8.2	10.7	0.239	0.133	0.106	55.8
		no	6	216	56	7.2	8.9	0.185	0.094	0.091	51.0
1.71[2]	5.42	yes	4	212	48	7.3	13.2	0.175	0.093	0.082	53.2
		no	5	235	63	3.2	11.8	0.144	0.061	0.083	42.0
2.63[2]	8.34	yes	6	176	23	5.4	12.8	0.148	0.074	0.073	50.1
		no	6	222	52	2.1	11.3	0.135	0.051	0.083	38.4

[1] The diets contained an average of 0.315% P.

[2] Phosphorus was supplied as H_3PO_4. In all other diets, it was supplied as a neutral equimolar mixture of KH_2PO_4 and K_2HPO_4.

[3] Calcium was supplied as $CaCO_3$ in all series except at the 0.43% Ca level in which $CaCl_2$ was used.

The effect of varying the amount of calcium in the absence of vitamin D was determined with a basal diet containing 0.3% P because numerous experiments in our laboratory had indicated that this level of P maintained serum P at a normal level (9 mg %). The additions of Ca increased growth, but not to the extent effected by the addition of vitamin D (table 2). However, when vitamin D was also given, Ca additions

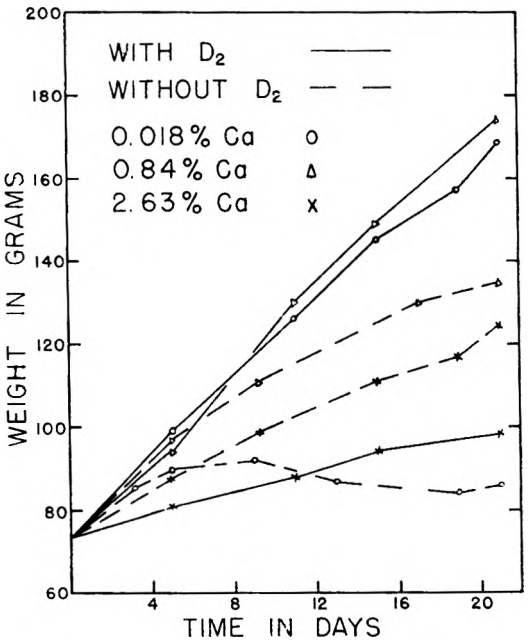

Fig. 2 Growth on diets containing 0.3% P and varying levels of Ca.

had only a minor effect. In fact, as the Ca intake reached a high level, the increase was less than that obtained with Ca alone; growth was then inhibited (fig. 2).

The additions of Ca never produced as beneficial an effect on bone as did vitamin D. At no level of Ca intake did the weight of the femurs, or their ash, equal that produced by the vitamin. Increase in organic bone, also in harmony with increases in body weight, was not equaled. The relations in percentage of ash however are not so decisive, for while

the effect of vitamin D was greater than that of Ca for each group, the maximum attained with Ca at a level of 0.43% in one group of 4 animals exceeded that obtained with a lower level when vitamin D was given. However, 20 rats on the same dietary regimen gave lower values. The width of the cartilaginous metaphyses of the distal ends of the radii and ulnae was always normal (0.25 mm), except for the non-D animals at the highest levels of Ca, namely, 1.7 and 2.6%. With these they were increased (1.80 mm). There was therefore a definite rachitogenic effect corresponding to the decrease in ash content such as is obtained with cereal grain rations containing 1.2% Ca.

When Ca was supplied as $CaCl_2$ or as a salt mixture resulting from the addition of H_3PO_4 to $CaCO_3$, the growth response was the same as that obtained with $CaCO_3$. Apparently the high basicity of the ration resulting from the use of the carbonate was not a determinant of the growth response.

In another series of experiments, the P content of the diet was increased progressively while the Ca was maintained at an adequate level (0.45%) throughout. The results (table 3) of this series reveal that the effect of vitamin D was not so great as in the earlier series (table 1) in which Ca was kept at the minimum level. While the inhibition of growth was reduced by the Ca the presence of vitamin D did not produce such heavy rats nor was the relative increase in weight so great as in the former series. Also the increase in serum inorganic P was not so great. All of these changes induced by the higher Ca intake were apparently effected by the increased Ca content of the serum.

A number of experiments were carried out in which the levels of dietary Ca and P were altered progressively after subnormal growth had been established on our basal vitamin D-free ration. The general plan of these experiments was to start a large number of rats on the desired diet. When the weight of the D-supplemented rats exceeded that of the non-D rats by 10 gm, most of the latter were given higher or

TABLE 3

Effect of varying dietary P content with adequate Ca intake [1] on growth, blood and bone

P CONTENT	Ca/P RATIO	D_2	FOOD INTAKE	GAIN IN WT.[2]	SERUM P	SERUM Ca	FEMUR			
							Dry wt.	Ash wt.	Organic wt.	Ash
%			*gm*	*gm*	*mg %*	*mg %*	*gm*	*gm*	*gm*	%
0.016	28.2	yes	233	34	4.6	12.7	0.110	0.041	0.069	37.5
		no	193	36	1.6	11.0	0.116	0.032	0.083	28.1
0.097	4.7	yes	282	72	7.4	11.5	0.153	0.068	0.084	44.5
		no	222	61	5.6	7.8	0.138	0.054	0.083	39.8
0.204	2.2	yes	299	92	8.6	10.9	0.210	0.115	0.095	54.4
		no	212	55	9.9	7.0	0.177	0.089	0.088	50.3
0.320	1.4	yes	294	100	9.8	11.0	0.233	0.130	0.102	55.8
		no	222	60	9.9	6.8	0.174	0.090	0.084	51.8
0.425	1.1	yes	267	84	10.3	11.0	0.215	0.123	0.092	57.1
		no	190	44	10.8	7.2	0.166	0.084	0.081	50.8
0.598	0.8	yes	288	94	11.2	10.9	0.217	0.122	0.095	56.2
		no	178	34	11.1	7.2	0.160	0.083	0.077	52.0

[1] The diets contained 0.454% Ca.

[2] Each group contained 4 rats.

lower dietary levels of Ca or P or both. Further dietary alterations were made when no growth response occurred, or when an initial response declined. Several animals were usually killed for an analysis of blood and bone whenever a dietary change was made.

In none of the above trials was the resultant growth equal to that obtained by the addition of vitamin D. Figure 3 presents the best response obtained. When P was increased

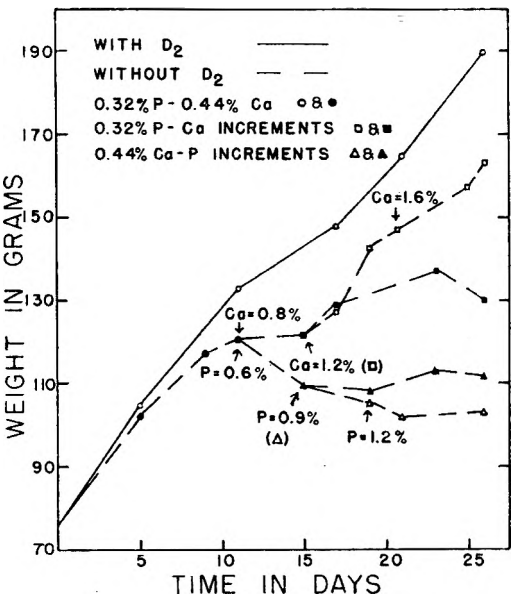

Fig. 3 Effect of successive dietary increments of Ca and P on growth.

above that of the initial dietary level of 0.3%, growth was depressed; when Ca was increased to levels of 1.2 or 1.6%, growth was increased. Later experiments showed that when the dietary Ca level was increased early in the experiment the effect was the same. Additional experiments with diets containing 0.4% Ca, with 0.4% P; or 0.3% P, with 0.8% Ca; with the Ca in the form of either $CaCO_3$ or $CaCl_2$, also failed to indicate that any change in the amounts or in the proportion of Ca to P could produce growth equal to that obtained with

vitamin D. The growth curves for one of these experiments, namely, those obtained with feeding a basal diet containing 0.4% P with 0.4% Ca, are found in figure 4.

As another device to secure better growth we availed ourselves of the technique of feeding the Ca and P-containing diets on alternate days to avoid reciprocal interference in their

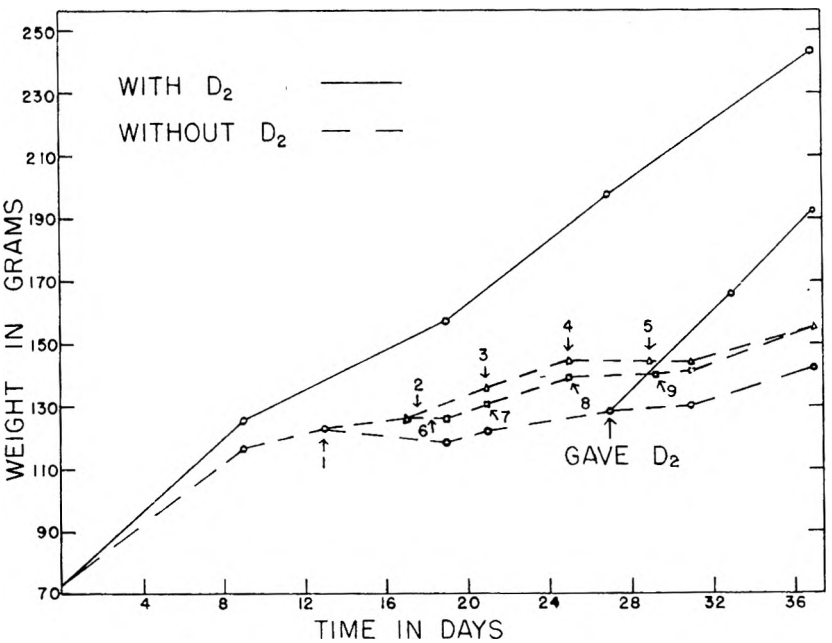

Fig. 4 Relation of growth response to dietary additions of vitamin D, Ca and P. Initial ration contained 0.4% P and 0.4% Ca. Numerals indicate following changes in ration: 1. 0.52% Ca, 0.4% P. 2. 0.65% Ca, 0.4% P; 3. 0.78% Ca, 0.4% P; 4. 0.78% Ca, 0.5% P; 5. 0.78% Ca, 0.6% P; 6. 0.52% Ca, 0.5% P; 7. 0.65% Ca, 0.5% P; 8. 0.65% Ca, 0.6% P; 9. 0.78% Ca, 0.6% P.

absorption. In these experiments, the Ca or P content of the respective diets was kept at the same levels as in the preceding experiments and in another case, they were doubled so that their intake on an equal calorie intake was not reduced. Again the resultant growth failed to equal that induced by a supplement of vitamin D.

Although early data of Bethke et al. ('23–4) and Bethke et al. ('32) suggested the potentialities of an assay method based on growth by means of which more than the mere presence or absence of the vitamin could be determined, only Coward et al. ('32) investigated its use. They obtained a logarithmic response of rats to graded amounts of vitamin D. However, a later report by Coward ('38) together with our own experience, based on a comparison with other methods, indicated that the difficulties of preparing the rats, inconsistent responses, and disappointing accuracy greatly limited the value of this technique.

Our present findings with diets low in Ca content suggested that a growth method of assay for vitamin D might, after all, be feasible, especially for samples low in Ca such as soft animal tissues. Limited experiments with low-Ca, high-P diets did indeed reveal a straight line relation of the logarithm of increases in body weight in three weeks plotted against the logarithms of a range of 6 to 525 I.U. of vitamin D given in 7 doses at three-day intervals. When comparable amounts were given in one dose, the net weight gains were less and growth was not so persistent. This is in harmony with the findings of Cruickshank and Kodicek ('53) who have reported an apparent rapid destruction of vitamin D after its administration. However, investigation of the sensitivity of a method of assay in which a low-Ca, high-P ration was used revealed decided limitations of such a technique. The advantage which had been gained in the operating range, over that obtainable with rations adequate in Ca content was lost in sensitivity. For example, while a single dose of 24 I.U. gave a growth differential of 20 gm on a diet adequate in Ca, the low-Ca rations gave a differential of only 8 gm.

DISCUSSION

If an analogy between the rat and the infant can be drawn from their growth response to vitamin D (Jeans, '51; Korenchevsky, '22; Mellanby, '49) the suggestion that rickets in the infant may at times be of the high-P, low-Ca type, is

supported. The not uncommon occurrence of fatty stools, which lead essentially to an increased fecal loss of Ca via the formation of Ca soaps (Shohl, '38) may in effect make the milk diet of the infant a high-P diet. In addition, the use of supplementary cereal foods, high in P, low in Ca makes this suggestion more tenable.

It would be expected that Ca was a limiting factor for growth with our high-P, low-Ca diets, and the addition of adequate Ca to such a diet confirmed this belief in so far as the non-D rats were concerned (table 2). However, the fact that additions of dietary Ca produced only one-half as much growth as that elicited by vitamin D alone, indicates that this vitamin performs a specific metabolic function other than has been shown heretofore. In the past, it has been assumed that vitamin D is primarily influential in the absorption and deposition of bone salts, but it is suggested by our results and those of Consuret ('54), Zetterstrom ('51), Zetterstrom and Ljunggren ('51) and Tulpule and Patwardhan ('54), that more consideration should be given to the possibility of a widespread effect on organic tissue metabolism of which increased growth is merely one manifestation. Possibly it induces a greater activity in the Krebs tricarboxylic acid cycle with a resultant increase in tissue weight and in tissue and urinary citric acid. This may explain the findings of increased citric acid in tissues (Freeman and Chang, '50; Steenbock and Bellin, '53) and its augmented excretion when vitamin D is given (Bellin and Steenbock, '52; Harrison and Harrison, '52; Bellin et al., '54). An increase in appetite (Consuret, '54) and gastric secretion and laxation (Herting and Steenbock, '55) while leading to increased growth may likewise be reflections of a basic effect on metabolic reactions.

The depression of growth by vitamin D with diets of a high Ca/P ratio has been attributed to a preferential shunting of the dietary P to bone, thus depriving the soft tissues of P which would otherwise be available for their growth (Schneider and Steenbock, '39). Jones ('44) however, correlated this inhibition with the hypercalcemia induced by vitamin D under

these dietary conditions. We observed a hypercalcemia with the three diets on which vitamin D depressed growth (tables 2 and 3); but we have no proof that this inhibition resulted from the hypercalcemia *per se*. To proye this theory we feel that the hypercalcemia should be invariable in its occurrence when growth is depressed.

While sera Ca and P were maintained at normal levels and life was maintained longer on the low-Ca rations when vitamin D was given (fig. 1), calcification of bone did not keep pace with the increase in body weight, and the bones became increasingly more thin-walled, porous, and congested as vitamin D stimulated growth. The question naturally arises: is the increased growth that we obtained by the addition of vitamin D to a low-Ca, high-P diet, a normal reaction? There has been some tendency to accept maximum growth as a primary desideratum and to use relative growth as an index of nutritional status. Thomson and Duncan ('54) have written recently: "The second lesson to be drawn from the evidence is that since the organism must be healthy to grow satisfactorily, growth is a good measure of general health, and since growth is the product of nutrition it measures nutritional status." That this statement may be invalid when applied to skeletal growth for short periods of time was indicated by the work of Zucker and Zucker ('46) which showed that an increase in bone weight or bone ash may be either the same, greater or less than the increase in body weight in nutritional deficiencies. Our data supporting this latter conclusion emphasize the necessity for caution in the use of maximum growth as an index of well-being.

On the basis of extensive data gathered in this laboratory over a period of years, we have found the normal levels of Ca and P in the blood serum of our young rats to approximate 10 mg % for Ca and 9 mg % for P. In the currently reported work, vitamin D tended to maintain serum inorganic P at a normal level regardless of the dietary Ca-P relationship.

For example, serum P was generally increased when dietary Ca/P ratios were high and decreased when they were low. The consistent elevation of serum Ca levels by vitamin D under all dietary conditions as contrasted with the variable effect on P suggests that this may be an important factor in determining the ultimate reaction to the vitamin.

SUMMARY

In a series of experiments with young rats, it was found that a low-Ca diet adequately supplied with phosphorus and other dietary essentials presented optimum conditions for eliciting the maximum growth differential which can be obtained with vitamin D. This effect of the vitamin was accompanied by a decrease in serum inorganic P, an increase in serum Ca, a decrease in the percentage of bone ash, an increase in the organic matrix of bone, and a slight increase in the width of the cartilaginous metaphyses. Vitamin D always tended to bring the serum P to a normal level. On the other hand, its only effect on the level of serum Ca was to increase it.

It was found impossible to duplicate the growth-promoting effect of vitamin D by varying the amount and proportion of P and Ca in the diet. The effect was the same, whether Ca was given as the carbonate or as the chloride. While the sole addition of Ca to a low-Ca, adequate-P diet increased growth, such additions when given continuously, or in successive increments, could not raise growth to the level induced by vitamin D alone. Also, when the dietary Ca/P ratio was raised too high, i.e. to an approximate ratio of 4.5 to 1.0, vitamin D depressed growth.

The large increase in soft tissue, as well as of organic bone, when vitamin D is given, suggests that it facilitates other reactions than those concerned with the intestinal absorption and the skeletal deposition of mineral elements. It appears that the weanling rat requires vitamin D for optimum performance.

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VITAMIN D AND GASTRIC SECRETION [1]

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In view of the increase in urinary pH (Steenbock et al., '51) which is obtained with a concurrent decrease in intestinal pH when rachitic rats are given vitamin D, the effect of this vitamin on gastric secretion becomes a matter of additional interest. Limited data on this subject, obtained with various rachitogenic diets and mostly with cod liver oil as the source of vitamin D, have appeared in the literature. With adult rats, on the Sherman-Pappenheimer diet, Abrahamson and Miller ('25) found that the gastric pH was lowered from 3.85 to 3.23. Later, Eastman and Miller ('35), using McCollum's diet 3143, reported no effect. Redman et al. ('27) with young rats on Zucker's rachitogenic diet reported a lowering of the pH from 6.5 to 4.3. Similarly, Eastman and Miller ('35) with young rats on this diet reported a reduction from 4.57 to 4.45 and from 4.81 to 4.64 with McCollum's diet 3143, but an increase from 3.93 to 4.26 with Steenbock and Black's diet 2965. Steenbock et al. ('51) also obtained some evidence of an increase in pH with a few rats on this latter diet.

It was quite evident that more extensive and more definitive data should be obtained. Eastman and Miller ('35) admitted that rickets was not evident in all of their rats; and, of course, a vitamin D deficiency is difficult to demonstrate in adult animals. Furthermore, other factors such as the diluting and

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buffering effect of food and of regurgitated intestinal juices have, hitherto, not been given any attention.

In our presently reported experiments, we instituted a regime of controlled feeding. This was followed by a short period of starvation, ligation of the pyloric sphincter, and collection of gastric secretion while the rats were denied access to water. This technique was essentially the same as that used originally with rats by Roe and Dyer ('39) as modified by several workers including Shay et al. ('45) and Hawk and Hundley ('52). It has been widely accepted in the study of gastric secretion, with particular reference to the prevention of gastric ulcers (e.g., see Pauls et al., '47; Funk et al., '52; Kowalewski et al., '54; Madden and Ramsburg, '51).

A gastric response to vitamin D would be of more than passing interest in infant nutrition because of the relatively low gastric acidity in the infant and its apparent reduction in rickets (Babbott et al., '23a, '23b; Zucker and Matzner, '23; Wills et al., '26).

EXPERIMENTAL

The investigations were carried out with young Sprague-Dawley rats weighing from 70 to 95 gm, but limited to a range of about 10 gm in individual series. They were kept on wide mesh screens and were fed, ad libitum, one of the following: our stock diet; our rachitogenic diet number 2965 of the usual composition, i.e., yellow corn, 76, wheat gluten, 20, $CaCO_3$, 3, and NaCl, 1 (Steenbock and Black, '25); or a variation of a basal semi-synthetic diet (Schneider and Steenbock, '39), which consisted primarily of glucose [2] 67; cooked egg white, 18, cottonseed oil [3] 10, roughage [4] 3, Ca- and P-free salts, 2, and a vitamin supplement (Bellin and Steenbock, '52). Optional supplements of various salts such as NH_4Cl, $NaHCO_3$, $CaCO_3$, and a neutral equimolar mixture of KH_2PO_4

[2] Cerelose.
[3] Wesson oil.
[4] Ruffex.

and K_2HPO_4 were added at the expense of the glucose content. When vitamin D was required by the experimental plan, 75 I.U. of calciferol in cottonseed oil were given orally with a syringe every three days. Distilled water was available to the rats except when gastric secretion was being accumulated. All rations were analyzed for Ca and P before use.

Diet 2965 was fed for 21 days. Since this produced approximately the same severity of rickets in 21 days as our semi-synthetic rachitogenic diet 23 produced in 9 days, as indicated by the width of the cartilaginous metaphyses [5], this latter feeding period was used for all experiments with purified diets. Food consumption and body weights were obtained every other day.

Following the induction of rickets, the rats were fasted for 16 to 24 hours, except those on the low-Ca, low-P diet. For these latter the fasting period was reduced to 10 hours to eliminate the incidence of tetany and fatalities in the "non-D" rats during the post-ligation period. Long fasting periods induced tetany even when 135 gm rats were used. The average loss in weight during the fasting periods ranged from 6 to 10% of the pre-fast body weights.

After fasting, the rats were anesthetized with ether, the pyloric sphincter was ligated, the abdominal incision was closed with a continuous stitch, and gastric secretion was allowed to accumulate for 6 hours. The abdomen was then reopened, the esophagus was clamped with a hemostat, the stomach was removed, and a blood sample was taken quickly by severing the carotid artery. The stomach contents were pressed out into a 10-ml graduated cylinder and measured. The gastric mucosa was examined for obvious perforations and blood clots. The pH of the secretion was determined with a Beckman Model G meter, and the stomach contents, plus the washings, were transferred to a 50-ml centrifuge tube and centrifuged. The volume of the residue was measured.

As a check on the rachitic state of the rats, serum inorganic

[5] We have used the term metaphyses to designate the cartilaginous area of the long bones which lies between the epiphyseal cap and the calcified diaphysis.

P was determined colorimetrically as described by Fiske and Subbarow ('25), and serum Ca by micro-titration of the oxalate with $KMnO_4$ according to Clark and Collip ('25). The ulnae and radii were removed, split, stained with a 1.5% solution of $AgNO_3$, and photographed for measurement of metaphyseal widths by projection. Bone ash was determined on the alcohol-ether extracted femora.

RESULTS

The collected data are shown in tables 1 to 4. The number of rats used to obtain the average values will be seen to differ widely. This was the result of rejections of data from rats which had ulcers or perforation of the stomach wall, blood in the secretions or large amounts of solid gastric contents — exceeding 0.5 ml in volume after centrifugation — as the result of coprophagy or the accumulation of foreign matter such as hair. All observed volumes were reduced to milliliters per square decimeter of body surface as calculated from body weights according to the formula of Diack ('30), using a K of 7.5. The weights used were those obtained immediately before starvation.

Vitamin D additions had a general tendency to increase the volume and acidity of gastric secretion as collected by our technique (table 1). In no case were the final values induced by this addition larger than those obtained with our stock diet. The effect of vitamin D therefore was to induce normalcy from the disturbed metabolic condition produced by our various vitamin D-deficient diets. It is evident that the largest increases in acidity were obtained with those diets which were low in P (23, 23-H, 34-B) or in which the availability of the P had been greatly depressed by an excess of Ca (2965, 11-U). When available P was present in near optimum supply (11-Q), with no depression of availability imposed by an excess of Ca, vitamin D had little effect. Furthermore the level of Ca in itself was not a factor as indicated by the results with two rations which were both low in P but which contained 1.24% Ca in one case

TABLE 1

Effect of vitamin D on volume and acidity of gastric secretion [1]

RATION	VITAMIN D_2	RATS [2]	VOLUME OF SECRETION PER UNIT BODY SURFACE [3]	CHANGE IN VOLUME WITH VITAMIN D_2	pH	CHANGE IN pH WITH VITAMIN D_2	ULCERATION [4]
			ml/dm²	%		%	*number of rats*
23 0.016% P, 0.45% Ca	—	19	1.63	+ 93	1.41	— 23	0 (24)
	+	21	3.14		1.09		0 (24)
23-H 0.017% P, 1.24% Ca	—	7	1.96	+ 62	1.33	— 14	1 (10)
	+	6	3.18		1.14		4 (11)
2965 Total P = 0.25% Ca = 1.29%	—	16	2.69	+ 26	1.17	— 10	4 (12)
	+	13	3.38		1.05		3 (9)
11-Q 0.31% P, 0.39% Ca	—	12	3.77	+ 10	1.16	— 3	0 (13)
	+	7	4.16		1.12		0 (12)
Stock 0.60% P, 0.77% Ca	+	5	5.36	..	1.04	..	5 (13)
11-I 0.59% P, 0.014% Ca	—	7	3.79	+ 34	1.13	+ 1	0 (12)
	+	8	5.08		1.14		0 (12)
34-B 0.016% P, 0.012% Ca	—	4	1.73	+ 84	1.46	— 14	0 (9)
	+	4	3.18		1.25		0 (11)
11-U 0.33% P, 2.56% Ca	—	9	2.50	+ 30	1.27	— 6	2 (10)
	+	4	3.26		1.19		7 (12)
23-G 0.015% P, 0.43% Ca 1.07% NH_4Cl	—	4	2.31	+ 38	1.28	— 14	1 (8)
	+	4	3.19		1.10		6 (12)
23-F 0.015% P, 0.47% Ca 10% $NaHCO_3$	—	7	2.31	+ 46	1.27	— 9	0 (11)
	+	8	3.38		1.15		1 (11)
23 0.015% P, 0.48% Ca Histamine injection	—	4	2.20	+ 70	1.24	— 10	3 (11)
	+	5	3.74		1.12		2 (12)

[1] The starvation period was 16 hours in all experiments except on ration 34B, with which it was 10 hours, and in some experiments on rations 23 and 2965, with which it was 24 hours. The secretion values after 16 and 24 hours were similar and were combined.

[2] Female rats were used with ration 23G and in some of the experiments with ration 23. When data from more than 8 rats on a ration are reported these represent average values of more than one series run in succession.

[3] Differences in the ml/dm² factor were significant at the 1% level on rations 23, 23H, 2965 and 11-I. The difference on ration 11-Q was insignifiant (according to the "t" test, Snedecor, G. W., "Statistical Methods," The Iowa State College Press, Ames, Iowa, 1946).

[4] Figures within parentheses give the number of rats examined for ulceration.

(23-H) and 0.012% in the other (34-B). The need for an adequate supply of P for normal gastric acidity was indicated further by the relatively high pH values obtained in the absence of vitamin D, and the absence of any effect when P was provided in great excess (11-I).

The changes in the volume of secretion, in general, paralleled those in acidity except for those obtained with the high-P diet (11-I). It is possible that the mechanism which is responsible for the increase in volume is not identical with that which increases the acidity. This differential may well have been brought out with this high-P ration because vitamin D, in contrast to its effect with diets low in available P, decreased the percentage of bone ash, increased the width of the metaphyses and decreased the level of blood P (Steenbock and Herting, '55).

The response to vitamin D apparently can be quite rapid. This was shown with rats which had received 525 I.U. in a period of three days after they had been kept on either diet 23 or diet 2965 for 9 and 21 days respectively. With the former, the gastric secretion was increased from 1.34 to 2.23 ml per dm^2 of body surface and the pH was reduced from 1.41 to 1.31. With the latter, vitamin D increased the volume from 2.36 to 3.12 ml per dm^2 and reduced the pH from 1.16 to 1.09. These results represent the average in each case of not less than 6 rats, all of which were severely rachitic.

The possibility that the trend in pH, if not the volume of secretion, might be altered by modifying the acid-base balance in the rachitic rat was tested in a limited way. Rats were fed diet 23 supplemented with 10% $NaHCO_3$ (diet 23-F) or with 1% NH_4Cl (diet 23-G) (table 1). The response of these rats to vitamin D resembled that obtained in the absence of these supplements.

Similarly, rats on diet 23 failed to deviate from their previously observed response to vitamin D, even when given a subcutaneous injection of 0.5 mg of histamine base in saline at the time of operation to augment secretory activity (table 1). When the dose was increased to 10.0 mg per 100 gm of

body weight, excessive ulceration, leading in some instances to perforation of the gastric wall, made the collections worthless. We did not pursue these trials further. Roe and Dyer ('39) used histamine to increase gastric secretion in rats, but Friedman ('43) claimed that this secretagogue had no effect.

The observation that a dietary deficiency in available P is correlatable with a decreased acidity of gastric secretion is

TABLE 2

Serum P and Ca

RATION	VITAMIN D_2	SERUM P [1]			SERUM Ca [1]		
		Before starvation	After starvation	After operation	Before starvation	After starvation	After operation
		mg %	*mg %*	*mg %*	*mg %*	*mg %*	*mg %*
23	—	4.0	7.5	13.8	11.4	8.5	8.9
	+	6.6	13.7	15.3	11.0	10.7	9.5
11-Q	—	9.8	12.3	16.0	9.7	8.9	10.0
	+	10.4	10.8	12.4	10.9	10.2	7.2
Stock	+	11.3	11.4	13.8	11.3	11.6	12.0
34-B	—	3.1	12.0	17.2	11.1	6.4	6.1
	+	10.9	15.0	19.3	11.2	8.3	8.2
11-U	—	5.0	9.5	13.4	11.5	8.5	7.0
	+	4.7	12.6	14.2	15.4	9.8	10.6

[1] Although data were obtained for all of the rations outlined in table 1 detailed presentation is limited to 5 as the results with them were considered adequately representative. The pre- and post-starvation analyses were usually made in two rats, but as many as 8 were used for post-operation analyses. The starvation period was of 16 hours duration except for ration 34-B with which it was limited to 10 hours.

supported by the values on serum P (table 2), bone ash and metaphyseal widths (table 4). In all cases but one (diet 11-U), the effect of vitamin D on secretion was accompanied by an increase in serum P as determined before the rats were fasted. We are inclined to disregard the one exception as the difference was marginal and as we have obtained consistent increases in other experiments with this diet. The serum P values bring out the additional well-known fact

(Kramer and Howland, '22; Park, '54) that starvation increases serum P in the rachitic rat. This increase was intensified by the operation. The fact that vitamin D affected gastric secretion in spite of these increases in serum P raises the question of whether the effect of vitamin D observed by us was possibly at a minimum.

In contrast to the correlation of serum P with secretory activity, a positive correlation of gastric pH with differences in serum Ca is not evident, probably because changes in serum Ca tended to vary less than those of P (table 2). The lower levels of serum Ca after the operation followed the general rule that serum Ca was reduced as the serum P was increased, but this reaction was not specific for individual groups. It is usually conceded (Babkin, '50) that hypercalcemia in dogs, whether induced by irradiated ergosterol, parathormone, or the injection of Ca salts, depresses gastric secretion. On the other hand, hypocalcemia following parathyroidectomy always increases secretion. Since variable changes in serum P would probably result from the use of the aforementioned techniques, it is conceivable that these may have been a disturbing factor in the investigations referred to.

We sought to prevent the increase in serum P in some rats by reducing the fasting period to 4 hours, as preliminary tests showed that 4 hours fasting was almost adequate to free the stomach of food residues. Other rats were given a solution of 33.0% glucose, together with 2.0% of our Ca- and P-free salt mixture and an addition of 0.8% Ca supplied as the chloride and gluconate, thereby providing them with both calories and salts other than P during the "fasting" period. These modifications were introduced in experiments with rats which previously had been kept for 10 days on diet 23, low only in P; diet 34-B, low in both Ca and P, or diet 11-I, low in Ca but excessively high in P.

The resultant data (table 3) revealed that under these modified conditions vitamin D had no effect on the gastric pH. Preceding the operation, that is immediately at the close

of the 4-hour period of fasting, the values for serum P were lower for both D and non-D rats than after a 16-hour fast. However, the increases in blood P were not prevented by the modified technique though the values were somewhat lower when vitamin D was not given than under the same circumstances after a 16-hour fast.

TABLE 3

Effect of shortening the fasting period from 16 hours (table 1) to 4 hours on the volume and acidity of the gastric secretion

RATION	VITAMIN D_2	NUMBER OF RATS	VOLUME PER UNIT BODY SURFACE	pH
			ml/dm²	
23 [1]	—	21	3.60	1.14
	+	15	3.38	1.15
34-B	—	7	4.02	1.15
	+	5	4.43	1.12
11-I	—	3	4.82	1.02
	+	5	5.80	1.04

[1] A sugar salt-solution was supplied to some rats for the first three hours of the fasting period and, with others, the gastric secretion was collected for three hours instead of 6 hours with no differences resulting under comparable circumstances.

Our results were not complicated by variable food consumption. In general, all diets were consumed readily, with little difference between the D and non-D groups except for three diets: viz., 11-I, excessively high in P and low in Ca; 11-Q, approximately optimal in Ca and P; and 11-U, excessively high in Ca. With the first two named, consumption was increased by vitamin D and on the third, it was reduced.

It followed, from the uniformity in food intake, that the weights of the rats at the beginning of the starvation period were practically the same for the D and non-D groups. Only with the three aforementioned diets were there outstanding differences (table 4). For 11-Q and especially with 11-I, the weights were increased by vitamin D; with 11-U, they were decreased. Not to be overlooked entirely is the slight depression in growth effected by vitamin D on the high-Ca, low-P diets which is in harmony with the recorded observa-

tions of Schneider and Steenbock ('39) and of Jones ('44). This decrease apparently was the result of the relatively high intake of Ca because it did not occur with diet 34-B in which the intake of both Ca and P was at a minimum level. This difference in Ca intake had no effect on the relative increases in volume or decreases in pH of the gastric secretion (diet 23 vs. 34-B).

TABLE 4

Body weights, and bone data

RATION	VITAMIN D_2	INITIAL WEIGHT [1]	WEIGHT BEFORE STARVATION [1]	FEMUR WEIGHT [2]	ASH	METAPHYSEAL WIDTH
		gm	*gm*	*gm*	%	*mm*
23	—	87	105	.1360	42.7	0.61
	+	87	95	.1520	46.6	0.13
23-H	—	89	96	.1310	44.9	0.55
	+	87	89	.1418	47.9	0.11
2965	—	87	111	.1494	43.9	0.78
	+	88	120	.2006	52.5	0.18
11-Q	—	75	103	.1569	50.7	0.28
	+	73	120	.1661	51.8	0.21
Stock	+	73	121	.1658	55.1	0.29
11-I	—	87	97	.1411	49.5	0.16
	+	86	127	.1507	47.9	0.26
34-B	—	72	111	.1110	37.2	1.36
	+	74	117	.1112	39.8	0.29
11-U	—	94	121	.1586	45.8	0.66
	+	92	97	.1645	49.1	0.16

[1] Body weights were limited to the average from rats for which gastric secretion data are presented.

[2] A minimum of 6 rats in each group was used for bone analyses.

It was recognized early by Dyer and Roe ('41) that the total volume which can accumulate in the stomach is necessarily limited by the amount of water which is available from other tissues. It became evident that vitamin D might have a pronounced effect on water metabolism. Not only was gastric secretion increased with all the diets fed, but Dr. John W. Cramer of this laboratory has observed a distinct polyuria when vitamin D was added to the low-Ca, low-P diet, 34-B and to the low-Ca, high-P diet 11-I. Furthermore, an incidence

of diarrhea has been observed occasionally in our laboratory when rats on experimental diets were given vitamin D.

Three experiments were carried out in which diet 23, low in P; diet 34-B, low in both Ca and P, and diet 11-I, high in P were fed to groups of 16 to 24 male rats weighing approximately 77 gm. These diets were selected because the effect of vitamin D with diets 23 and 34-B contrasts sharply with that on diet 11-I (table 1). Growth, food consumption, water intake and urine output were measured over a two-week period. The rats were then killed with ether, either immediately or following a 16-hour fast. A wrist and femur were removed for analysis from each rat, and the carcasses were cut open and dried at 105°C. The femora were extracted and ashed, and the distal cartilaginous metaphyses of the radii were measured. To economize space, the results of these experiments will not be presented in detail; they did not reveal any convincing correlation between body water, urinary volume and gastric secretion. The changes induced by vitamin D in relation to body weight, femur ash and metaphyseal widths, corresponded to those presented in table 4. With diet 34-B for example, vitamin D increased body weight 12%, water consumption 45% and urinary volume 90%, but body water remained practically constant, 72.5% vs. 71.9% for the non-D rats. With diet 23, the corresponding water content was 72.8% vs. 72.4% and with diet 11-I, 71.8 vs. 73.2%.

It is evident that the increase in gastric secretion cannot be attributed to a greater degree of body hydration. With reference to polyuria, there remains no question of its incidence with diet 34-B. It was accompanied by a definite polydipsia. These reactions were not obtained with diets 23 and 11-I. It is of interest that a marked polyuria and polydipsia usually are also associated with the hypercalcuria and hyperphosphaturia of hyperparathyroidism (Albright and Reifenstein, '48).

Our data on the occurrence of ulcers in different groups (table 1) are probably too limited to warrant more than passing comment; but it may be significant that ulcers were

not produced with diets 23-F, 11-I, 11-Q, and 34-B, which ranged from low (.014%) to moderate (.45%) in Ca content. On the other hand, ulcers were produced with diets in which the Ca was increased to 0.77% or more. These ulcers were usually found in the rumen but occasionally in the antrum. At the highest levels of Ca intake, vitamin D appeared to increase their incidence. These data are not in accord with the conclusions of Zucker and Berg ('43, '44) or Zucker et al. ('45) obtained under somewhat different experimental conditions that low-Ca diets tend to produce ulcers and that vitamin D reduces this tendency. Nasio ('45), with cincophen-treated dogs, has reported that vitamin D_2 frequently prevented or healed ulcers.

In view of the dependency of a demonstrable gastric effect on the starvation-pyloric-ligation technique, an application of our results to the intact rachitic rat can be made only with reservations. However, it appears that vitamin D, when given with rachitogenic rations low in available P, may increase the volume and acidity of gastric juice. These increases in volume and acidity may not only be factors in antirachitogenesis but also in the absorption of Mg (Meintzer and Steenbock, '55) and Pb (Sobel and Burger, '55).

SUMMARY

The effect of vitamin D on gastric secretion was determined in a series of experiments with rats kept on natural or semi-synthetic rations, normal or rachitogenic. The gastric secretion was collected after a 10- to 24-hour period of fasting followed by pyloric ligation. Vitamin D was found to increase the volume and acidity of the secretion after the feeding of rachitogenic diets. No increase in acidity was obtained with rats on a normal diet or on a diet high in P. When induced with rachitogenic diets the increase could be correlated with an increase in serum P. No effect of vitamin D was demonstrable when the preoperative fasting period was reduced to 4 hours. Analysis of the rats failed to reveal any effect of vitamin D on body hydration.

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PROTEIN AND AMINO ACID REQUIREMENTS OF THE GUINEA PIG

I. EFFECT OF CARBOHYDRATE, PROTEIN LEVEL AND AMINO ACID SUPPLEMENTATION [1]

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TWO FIGURES

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Early attempts to develop an adequate purified diet for the guinea pig probably failed because the diets were inadequate in bulk (Booth et al., '49; Woolley and Sprince, '45), potassium and magnesium (Roine et al., '49) and casein (Woolley and Sprince, '45; Booth et al., '49). As the guinea pig is herbivorous, and normally eats bulky foods rich in potassium and magnesium, the need for these substances is not surprising. Although a more satisfactory purified diet for guinea pigs has recently been developed (Reid and Briggs, '53), the need for high levels of casein has not been adequately explained.

Woolley and Sprince ('45) noted that the growth rate of the guinea pig was improved when the casein content of the diet was increased to 37% [2] and reported that a combination of arginine, cystine and glycine added to 22% [2] casein diets gave a growth response equal to that obtained with the extra casein. However, the best growth observed by them was 3.9

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the National Livestock and Meat Board.

[2] Fiber and artificial bulk materials have been considered as nutritionally inert and all values have been recalculated on this basis. For example, the standard 30%-casein, 15%-bulk ration contains 30/85% casein = ca 35% casein.

gm/day, only slightly better than half the normal growth rate for the young guinea pig. Booth et al. ('45) obtained no response to arginine, cystine, and glycine. These workers also tried replacing sucrose with dextrin, and found no improvement.

Work done in the past few years has indicated that dextrin is superior to sucrose as the carbohydrate for young rats fed low casein diets and for chicks fed higher levels of casein (see Monson et al., '54 for references). In view of these results and of the fact that the previous conclusions of Booth et al. ('45) were drawn from only one experiment, it seemed worthwhile to study the protein requirements of the guinea pig and to reinvestigate the effect of the type of the dietary carbohydrate on the protein requirement.

EXPERIMENTAL

Adaptation and pre-experimental period. Male guinea pigs of mixed strains and weighing 160 to 200 gm were obtained from a dealer [3] and adapted to the synthetic diets before being placed on experiment. During this adaptation period the guinea pigs were fed either the normal purified diet or a mixture of the various experimental diets to which 20% of alfalfa meal had been added. A few lettuce leaves were given during the first few days until the guinea pigs had definitely started to eat the adaptation ration. The amount of alfalfa meal was gradually reduced until the guinea pigs gained weight on the purified diet alone. Guinea pigs were weighed three times weekly and were considered to be adapted when about 80% of the animals gained weight over a 4-day period. The guinea pigs were then divided into groups of 5 animals having the same average starting weight (ca 180 gm). Since adapted guinea pigs will grow fairly uniformly for at least 12 weeks, the initial variation of 30 to 40 gm did not affect the results which are based upon average growth rates in grams/day rather than on total weight changes or starting and finishing weights.

[3] Gopher State Caviary, St. Paul, Minnesota.

The guinea pigs were housed initially in 1/4″ mesh cages with raised screen bottoms. At a weight of about 300 gm the animals were transferred into 1/2″ mesh cages. Cages and the rack were changed and sterilized at least once a month and the cages more often if they became contaminated with feces. The shavings beneath the cages were changed three times weekly for young guinea pigs and daily for older animals.

Diets were mixed at least once every two weeks, and were refrigerated. Special metal self-feeders attached to the front of the cage, minimized food wastage and prevented contamination of the diet with feces and urine. The animals were fed daily and the feed trough was checked twice daily to insure that the supply of ration was ample. The animals were given at least one fresh bottle of water daily, supplemented with 35 mg of ascorbic acid. Older and heavier animals often required two bottles a day. All empty water bottles and tips were thoroughly cleaned daily in a detergent solution containing a disinfectant. In our experience the importance of having fresh water and ration before the guinea pigs at all times and observing strict sanitary procedures can not be overemphasized.

Diets and experimental plan. The composition of the basal 10% casein diet is given in table 1. Increases in the level of casein, and the addition or omission of amino acid supplements were made at the expense of carbohydrate.

The vitamin mix provided in milligrams per 100 gm of ration: inositol 200, niacinamide 20, PABA 10, Ca-pantothenate 8, riboflavin 3, thiamine-HCl 2, pyrodoxine-HCl 2, folic acid 1, in micrograms: biotin 100, vitamin B_{12} 4; fat soluble vitamins were provided weekly (or more often if shorter experimental periods were used) by dropper in the following amounts: A, 2000 I.U.; D, 20 I.U.; E, 12 mg; K, 0.2 mg.

The original experimental plan was to measure growth rates of groups of guinea pigs receiving graded levels of casein (10 to 30%) for 4 weeks, after which the livers would be analyzed for fat. However, the 10 and 15% casein groups,

in which fatty livers might have been expected, were discontinued after two weeks. After the 4-week period, urea and amino acid supplementation were tried with the 20 and 25% groups, respectively.

The marked responses to amino acid supplementation, together with the marked growth differences between the 30% casein groups and the 20 and 25% groups during the first part of the experiment suggested that certain amino acids were limiting when lower levels of casein were fed. Consequently,

TABLE 1

Basal diet

	%
Sucrose or dextrin	78.00
Casein (alcohol extracted)	10.00
Corn oil	4.00
Salts IV [1]	4.00
Potassium acetate	2.50
MgO	0.50
Choline chloride	0.35
DL-methionine	0.30
Vitamin mix	0.25
DL-tryptophan	0.10
	100.00
Roughage [2]	18.00

[1] Hegsted et al. ('41).

[2] Solka-floc, Brown and Company, Berlin, New Hampshire.

all 6 groups were used to study the effect of amino acid supplementation during consecutive, short-term (4 to 8 days) periods.

The experimental plan of amino acid supplementation is summarized along with the results in table 2. Figures 1 and 2 show in detail the results and the experimental plan for the 20 and 30% casein-sucrose groups.

RESULTS

No data are presented for the groups that received 10 and 15% of casein. As these animals steadily lost weight,

and some died during the second week, they were discontinued after two weeks.

The figures in the first two lines of table 2 show that there was no significant growth difference between groups receiving sucrose or dextrin with 20 or 25% of casein. The rate of gain of these groups was 1 gm/day or less with no supplements and was only very slightly improved when the diets were supplemented with methionine and tryptophan. In contrast,

TABLE 2

Summary of effect of dietary carbohydrate, level of protein, and of amino acid supplementation on the growth of guinea pigs fed purified diets [1]

CARBOHYDRATE	SUCROSE			DEXTRIN		
% OF CASEIN	20	25	30	20	25	30
SUPPLEMENT						
None	— 0.5	1.0	5.4 [2]	— 0.2	0.8	5.4 [2]
0.3% DL-methionine 0.1% DL-tryptophan	1.0 [4]	1.6 [4]	3.6	1.2 [4]	1.6 [4]	5.6
0.5% L-arginine-HCl	4.9	4.6	6.7 [2,3]	6.0 [2]	5.3 [2]	3.9 [2,3]
0.5% L-arginine-HCl 0.3% DL-methionine	5.0	6.2	..	5.7	5.6 [2]	..
0.5% L-arginine-HCl 0.1% DL-tryptophan	..	5.0	5.1	..	5.0	2.8
0.5% L-arginine-HCl 0.3% DL-methionine 0.1% DL-tryptophan	6.9 [2]	6.4 [2]	..	5.3 [2]	6.0	..
0.5% L-arginine-HCl 0.3% DL-methionine 0.1% DL-tryptophan 1.0% glycine	6.5 [3]	4.3	..	7.1 [2]	5.3	..
0.3% DL-methionine 0.1% DL-tryptophan 4.2% urea	0.1	..	..	2.0	..	..

[1] Values represent typical rates of gain in grams/day for periods averaging 5 days.

[2] Average of the two most representative periods.

[3] 0.3% L-arginine-HCl.

[4] Results from the first 4 weeks.

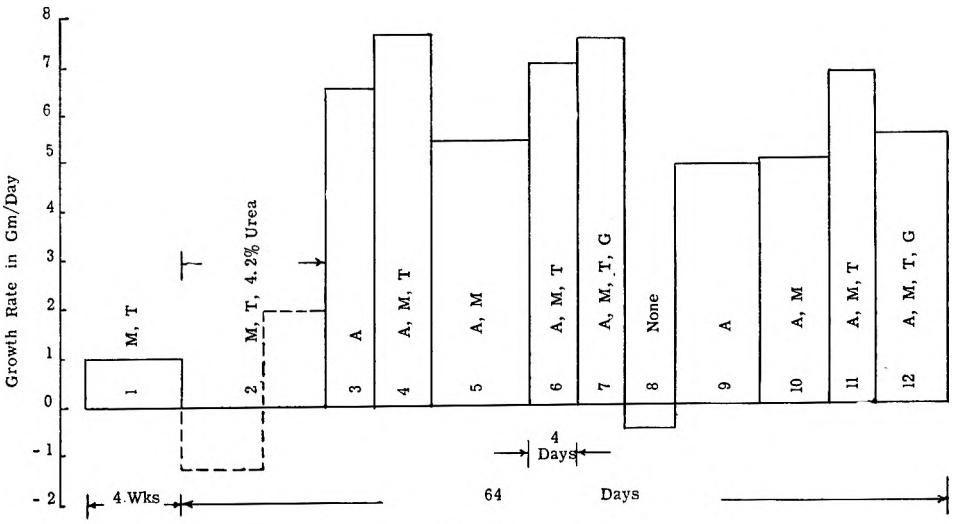

Figure 1

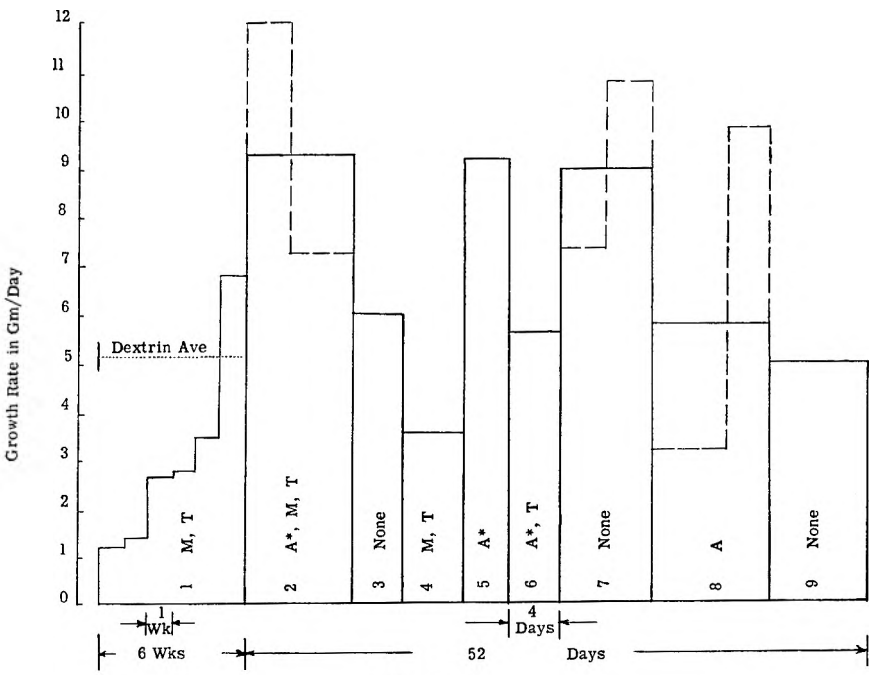

Figure 2

Figs. 1 and 2 Growth responses to amino acid supplementation of male guinea pigs receiving 20% casein-sucrose (fig. 1) and 30% casein-sucrose (fig. 2) diets.

Key to supplements: M = 0.3% DL-methionine; T = 0.1% DL-tryptophan; G = 1.0% glycine; A = 0.5% L-arginine-HCl; A* = 0.3% L-arginine-HCl. Dashed lines indicate responses within the period. Average weights at end of experiment: 20%, 517 gm; 30%, 678 gm.

the groups receiving 30% of casein alone grew at a rate greater than 5.0 gm/day Supplementation of the 30% casein diets with methionine and tryptophan caused no growth stimulation and actually appeared, initially, to retard the growth of the sucrose group.

Supplementation with arginine markedly stimulated the growth of all animals except those in the 30% casein-dextrin group, in which case arginine appeared to inhibit growth. The inclusion of methionine with arginine produced a further response only in the 25% casein-sucrose group. Both tryptophan and methionine were necessary before a further response was obtained with the 20% casein-sucrose group.

When the individual histograms of figures 1 and 2 are examined, a number of details can be noticed that are not apparent from the summary table. Details of the other groups will be discussed in the text.

In contrast to the similarity in the growth responses of the groups fed either sucrose or dextrin with the lower levels of casein, there was a marked difference during the early part of the experiment between the sucrose and dextrin groups that received 30% of casein supplemented with methionine and tryptophan. The animals of the dextrin group grew about 5 gm/day for the entire 6 weeks as compared to the initial response of less than 2 gm/day for those of the sucrose group. However, the superiority of the dextrin group over the sucrose group gradually decreased until at the end of 6 weeks, the sucrose group was growing slightly more rapidly than the dextrin group. During the last two weeks, the rate of growth of both of the 30% casein groups was far superior to that of the groups fed the lower levels of casein.

At the end of the initial 4-week period, 4.2% of urea, equivalent in nitrogen to 15% of casein, was added to the 20% casein diets, and arginine and threonine were added to the 25% casein diets. With 20% of casein, the dextrin group continued to gain slightly during the first week after the addition of urea whereas the sucrose group lost weight. There

was less difference between these two groups during the second week, when the sucrose group started to gain weight.

In contrast to the lack of a significant response to urea supplementation in the 20% casein groups, amino acid supplementation produced very striking growth responses in the groups fed 25% of casein. As a consequence all 6 groups were used thereafter to study the effects of amino acid supplementation.

Arginine. As noted before, with the exception of the 30% casein-dextrin group, arginine supplementation significantly improved the growth rate of animals in all groups. This was particularly evident with the 20 and 25% casein groups as can be seen by comparing lines 1 and 3 in table 2. Periods 3 and 9 of figure 1 show that the effect of arginine was immediate and very striking. Table 2 also shows that a definite response to arginine was obtained with the 30% casein-sucrose group. This is more clearly demonstrated in figure 2 (periods 2 and 5). The growth rate during the first part of period 2 was 12 gm/day. This initial surge of growth when arginine was first given was also noted in the 20% and 25% casein groups. This type of growth response which appears to be compensatory, was also observed when growth-depressing levels of amino acids were removed.

The peculiar response of the 30% casein-sucrose group to 0.5% of arginine in period 8 (fig. 2) is of interest. Apparently, the addition of 0.5% of arginine was excessive and caused an initial depression in the growth rate. However the group did adapt to this level and grew at the compensatory rate of 9.8 gm/day during the second part of the period to give an average rate greater than that recorded when arginine was omitted during period 9. This example illustrates the extreme sensitivity of the guinea pig to dietary changes. This dietary sensitivity often results in guinea pigs refusing to eat altered rations, especially if an essential nutrient is taken out, or if a component is added in excess. Thus from periods 2 and 5 (fig. 2) it would seem that 0.3% of arginine is the maximum amount that can benefit the young guinea pig re-

ceiving 30% of casein with sucrose and that the 0.5% of arginine received in period 8 is excessive.

Even though an initial response to arginine, methionine, and tryptophan was observed with the 30% casein-dextrin group, the response declined during the second part of the period, giving an average which was only slightly better than the rate observed during the preceding period with methionine and tryptophan. In contrast to the positive growth responses to arginine alone with all other groups, there appeared to be some depression of the growth rate of the 30% casein-dextrin group during such periods.

Methionine. Although no consistent response to methionine was observed with the dextrin groups, the addition of both methionine and arginine to the diet of the 25% casein-sucrose group produced a greater growth response than did arginine alone as can be seen from table 2. In contrast to the positive response to methionine with the group receiving the 25% casein-sucrose diet containing arginine, no similar response was obtained with the 20% casein-sucrose group.

A period in which 0.3% L-cystine was substituted for methionine in the 25% casein diets, showed that the response to cystine with the sucrose group was just as good, if not slightly better than responses to methionine, during corresponding periods. In the dextrin group, however, the response to cystine was somewhat less than the response to methionine. No quantitative comparison between cystine and methionine can be made from these preliminary results.

Tryptophan. Apparently tryptophan is more limiting than methionine for guinea pigs fed 20% of casein with sucrose since no further response was obtained until both tryptophan and methionine were added to this diet as seen in table 2 and figure 1. In contrast, no definite response to tryptophan supplementation, above that to arginine and methionine, was evident with the 25% casein-sucrose group. The response to arginine and tryptophan, compared to the response to arginine alone, and preliminary data from longer term experi-

ments, also indicate that guinea pigs receiving a 25% casein-sucrose diet do not need supplementary tryptophan.

The sensitivity effect, previously noted with excess arginine, was also seen with excess tryptophan. Thus supplementary tryptophan appeared to be excessive and inhibitory for the 30% casein-sucrose group. Responses to methionine and tryptophan (period 4, fig. 2) and to arginine and tryptophan (period 6), as compared to the responses in the preceding and following periods, suggest that there was some depression of growth during periods 4 and 6. The fact that arginine appeared to be of some benefit to the 30% casein-sucrose group would indicate that tryptophan caused the decreased rate of growth in period 6. Whether supplementary methionine had any modifying action on the effect of excess tryptophan can not be determined from these data.

Supplementary tryptophan appeared to be without effect in the 20 and 25% casein-dextrin groups. The 30% casein-dextrin group responded poorly to arginine and tryptophan, probably because of the growth-depressing effect of arginine. Although further inhibition from tryptophan can not be ruled out, the combination of methionine and tryptophan did not produce any growth depression of the 30% casein-dextrin group.

Glycine and threonine. Glycine in combination with arginine, methionine and tryptophan was tried twice with the 20% casein groups and once with the 25% casein groups. Owing to inconsistent responses, no definite value of glycine supplementation was evident with the 20% casein-sucrose group. However, the 20% casein-dextrin group gave definite responses during both periods. It is interesting that both non-specific sources of nitrogen gave slightly better responses with the 20% casein-dextrin group. Both of the 25% casein groups showed some decrease in growth rate when 1% of glycine was added to diets already containing supplementary arginine, methionine and tryptophan. This effect was slightly more pronounced with the sucrose group.

DL-Threonine (0.2%) was tried only once and then in combination with the three other amino acids with the 25% casein groups. Supplementary threonine would appear to be unnecessary since all groups grew rather well during other periods without threonine.

DISCUSSION

The results of these experiments clearly show that levels of casein of 25% or less are inadequate for the young, rapidly-growing, guinea pig Although rats fed only 10 to 15% of casein grow quite well, guinea pigs fed these levels of casein, supplemented with methionine and tryptophan, do not even survive. A level of 30% of casein is required for satisfactory growth and from previous work, 35% of casein seems to be somewhat superior.

The very striking responses obtained when arginine was added to the diets containing the lower levels of casein, indicate that it is the most limiting amino acid in casein for the young guinea pig. Even the 30% casein-sucrose group responded to arginine supplementation. It is also evident that methionine is limiting in the 25% casein-sucrose diets supplemented with arginine and that both tryptophan and methionine are limiting in the 20% casein-sucrose diet containing arginine, tryptophan apparently more so than methionine.

Much of the difficulty in raising guinea pigs on purified diets obviously stemmed from the fact that the early diets contained only 20% of casein. Although, guinea pigs grow very well on diets of natural materials [4] which contain only 20% of protein,[5] a comparison of the amino acid composition of plant proteins with casein shows that there is usually more arginine in the former. Some plant proteins, particularly those of certain grasses, contain as much as 8% of arginine, about twice the amount found in casein. As the guinea pig is herbivorous, it is conceivable that it has evolved under

[4] Rockland guinea pig pellets.

[5] See footnote 2, page 483.

conditions in which its needs for arginine, in comparison to the rat, have been met more from dietary sources and less from internal synthesis.

In addition to the fact that casein is relatively low in arginine, it also appears that the arginine in casein is not completely available to the guinea pig. A diet containing 35% of casein would provide, theoretically, about 1.22% of arginine while by microbiological assay,[6] commercial guinea pig pellets [7] contain 0.94% of arginine.[8] If the amount in pellets is assumed to be the amount needed and utilized, the arginine in casein is only 77% available. Since the previous assumptions are probably too liberal, it is doubtful whether the arginine in casein is more than 70% available for the young guinea pig. This finding is similar to that of Arnold et al. ('36) who found the arginine in casein to be only partially available for the chick. Thus the relatively high requirement of the guinea pig for arginine, together with the relatively low level and limited availability of the arginine in casein, makes it necessary to use 35% of casein (30% on the old basis) in purified diets for the young guinea pig. Rough calculations from this information would suggest that the requirement of the young guinea pig for arginine is approximately 0.8% of the diet, a value considerably in excess of that of the young growing rat, and approaching that of the chick.

The slightly greater responses of the 20 and 25% casein-dextrin groups to arginine alone are of questionable significance. These responses and the fact that no additional responses were obtained from methionine or tryptophan or both could be interpreted as a possible specific amino acid sparing action by dextrin as has been observed with rats.[9] However, it should be emphasized that the responses to *combined* amino acid supplementation were usually greater with the sucrose

[6] Determined through the kindness of Dr. E. W. Lewis and F. N. Hepburn.

[7] See footnote 4, page 493.

[8] See footnote 2, page 483.

[9] Heinicke, H. R., A. E. Harper and C. A. Elvehjem, unpublished data.

groups and that no definite conclusion as to a possible sparing action can be drawn at this time.

It is apparent that under the conditions of these experiments, dextrin did not cause a marked improvement in the growth of guinea pigs receiving diets inadequate in protein, as has been observed with the young rat (Harper and Katayama, '53). It should be pointed out that our conditions did not permit an intermediate rate of growth, with which such an effect is most likely to occur. Although still in the rapidly-growing stage, the guinea pigs were about 8 weeks old when amino acid supplements were started. Since the protein sparing action of dextrin with rats decreases as the animals grow older, it would be interesting to study the effect of dextrin on the growth of very young guinea pigs receiving levels of protein and amino acids that permit an intermediate rate of growth.

Whether the differences that were observed between the dextrin and the sucrose groups are related to a possible action of dextrin in slowing the rate of passage of feed through the gastrointestinal tract (Monson et al., '50; Stokstad et al., '53) is not known. The sparing action of dextrin for young rats may in some way be related to the more bulky nature of dextrin, as compared to sucrose. If this were the case, then the large amount of bulk already present in guinea pig diets would probably decrease any such effect from dextrin.

SUMMARY

1. The need for high levels of casein in purified diets for guinea pigs has been shown to be due to the need for a few specific amino acids rather than for protein *per se*.

2. The most limiting amino acid in casein for the young guinea pig is arginine, apparently owing to a relatively high requirement of the guinea pig for arginine and to the relatively low level and limited availability of the arginine in casein.

3. With arginine supplementation, methionine becomes limiting in 25% casein-sucrose diets and both tryptophan and methionine become limiting in 20% casein-sucrose diets.

4. The guinea pig was shown to be extremely sensitive to dietary changes, such as the omission of an essential item or the addition of a component in excess.

5. No definite protein sparing action was found with dextrin, and the differences between sucrose and dextrin diets that were observed were usually not marked. In general, the responses to combined amino acid supplements were slightly greater with sucrose diets but in a few specific instances dextrin appeared more effective.

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TRANSFER OF PHOSPHATE IN THE DIGESTIVE TRACT

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Classical studies of the chemistry of digestion have established that there is an exchange of minerals between the gastrointestinal contents and the blood. Bergeim ('26a) developed a method using ferric oxide as an inert reference substance, or tracer, to determine the extent and site of this exchange. In rats, Bergeim ('26b) found that the secretion of endogenous phosphorus occurred mainly in the middle segment of the small intestine and might amount to 100 to 200% of the daily food phosphorus. There was evidence of phosphorus absorption in the cecum in most cases, and in the colon in all cases.

Thus, digestion is accompanied by a cyclic process in which ions are added in the upper portions of the digestive tract, presumably as part of the digestive juices, and withdrawn in lower portions, resulting in an "internal circulation of the minerals" (Shohl, '39). Information on this mineral exchange is of nutritional importance (Kleiber et al., '51), particularly with regard to phosphorus, which is frequently a limiting factor in nutrition.

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The older chemical methods, including the use of inert reference substances, are limited in investigations of exchange because they measure only net changes and are often insensitive to the total exchange or underestimate its extent. Isotopic tracers, however, make it possible to follow the distribution of a particular quantity of some element in the animal body, and by comparing this distribution at various times after administration of the tracer, the nature and rate of exchange processes can be evaluated. In the experiments reported here, radiophosphorus (P^{32}) has been used to label the circulating phosphate in swine, and the appearance of this material in the gastrointestinal tissues and contents of sacrificed animals has been used to evaluate the transfer of phosphate in the digestive tract.

METHOD

Swine used for this study were of three age groups: 2, 4, and 8 months, containing respectively, 3, 4, and 3 animals. The mean body weights of these groups were: 15.8 $\pm$ 1.9 kg, 30.9 $\pm$ 1.8 kg, and 90.2 $\pm$ 1.8 kg, corresponding, respectively, to 5, 20, and 50% of the mature body weight. A buffered isotonic solution containing radiophosphate was injected into a marginal ear vein of each animal and they were then returned to their usual routine until slaughter, which occurred from one to 48 hours after injection. Samples were taken of the tissue and contents of the stomach, small intestine (at the midpoint and posterior to the entry of bile and pancreatic juice), cecum, colon and rectum. In the case of the stomach the epithelial tissue was stripped from the underlying musculature, and in the case of the rectum the heavy sphincter muscle was separated prior to processing the sample. The other intestinal tissues were merely freed of fascia, fat and residual contents and processed as the entire tissue.

Determinations of dry matter, phosphorus and radioactivity were made on each sample. The experimental swine and

analytical procedures used have been previously described (Smith et al., '51).

RESULTS AND DISCUSSION

The results of the chemical analysis of the tissues and contents of the digestive tract are summarized in table 1 as the means and their standard deviations for each age

TABLE 1

Dry matter and phosphorus concentration in tissues and contents of the gastrointestinal tract of swine (mean and its standard deviation)

	2-MONTH SWINE		4-MONTH SWINE [1]	8-MONTH SWINE	
	Tissue	Contents	Tissue	Tissue	Contents
Dry matter (%):					
Stomach	17.3 ± 1.1	14.0 ± 4.9	16.8 ± 1.0	18.8 ± 1.3	12.6 ± 4.1
Small intestine	16.7 ± .6	5.8 [2]	17.2 ± 2.0	16.9 ± .5	6.1 ± 2.6
Cecum	20.6 ± .8	9.4 ± .3	21.1 ± 1.3	25.7 ± 1.3	16.5 ± 10.0
Colon	19.8 ± 1.5	18.8 ± 3.5	22.8 ± 2.6	27.1 ± 1.5	18.2 ± 2.9
Rectum	25.6 ± 1.5	20.2 ± 1.7	24.5 ± 2.5	38.7 ± 1.4	20.7 ± 2.7
Phosphorus (mg P/gm dry matter):					
Stomach	11.1 ± .6	4.1 ± .6	12.6 ± .5	8.3 ± .7	2.9 ± .7
Small intestine	13.8 ± .7	5.7 ± 2.3	11.0 ± .5	10.0 ± .8	7.2 ± 4.2
Cecum	7.1 ± .1	6.8 ± 3.1	6.7 ± .3	4.3 ± .6	5.5 ± 2.1
Colon	8.3 ± .3	9.4 ± 5.9	6.1 ± 1.0	4.9 ± .4	12.8 ± 4.6
Rectum	5.6 ± .2	8.1 ± 5.1	4.5 ± .7	2.8 ± .2	12.8 ± 4.8

[1] Autopsy of the 4-month-old swine showed their gastrointestinal contents to be contaminated with large amounts of sand. On analysis, samples of the digesta were observed also to have a high dry matter and phosphorus content. Since this condition was nutritionally abnormal, data concerning the contents of the digestive tract of the 4-month-old swine were omitted from this report.

[2] One determination only.

group. Both the water and the phosphorus (per unit of dry matter) contents of the tissues are high in the anterior portion of the digestive tract and decrease lower down. Such progressive changes, or gradients, in composition have been discussed by Alvarez ('40), particularly with regard to functional activity gradients along the gut. Composition gradients of the tract masculature were prediteed by Alvarez as the basis of gastrointestinal dynamics, and similar gra-

dients of the mucosa, associated with the digestive and absorptive activity. The relationship between water content and metabolic rate of tissues (De Robertis et al., '54) and the essential role of phosphate in cellular metabolism would suggest that the chemical composition gradient observed in the digestive tract of swine is associated with an energy metabolism gradient which decreases in the lower part of the tract. The composition of the gastrointestinal contents, as might be expected, is much more variable than that of the tissues. The water content of the digesta was similar to that of the tissues, being highest in the small intestine and decreasing lower down. The phosphorus concentration of the digesta, however, was the reverse of that of tissue; it was quite low in the upper portions of the digestive tract and increased lower down.

Age seems to affect the composition of the tissues. Generally, the water content decreases with increasing age except in the stomach and small intestine, and the phosphorus concentration also decreases. No consistent age affects are evident in the composition of the gastrointestinal contents.

The results of the chemical and radiological analyses of the various samples were used to calculate their specific activities.[4] The specific activity values have been "standardized" for the variables of body size and tracer dose administered, the resulting figures being termed "standard specific activity."[5] Table 2 contains the standard specific activities

[4] Specific activity = $\mu c\ P^{32}$/mg P; 1 μc = 1 microcurie = 2.22×10^6 disintegrations per minute.

[5] Standard specific activity = $\dfrac{\mu c\ P^{32}\text{/mg P}}{\text{mc } P^{32}\text{ inj./kg body weight}}$; 1 mc = 1 millicurie = 1000 microcuries.

The deposition of a radioisotope is also frequently expressed in the literature as: "per cent of injected dose per milligram element," or "per cent of injected dose per gram tissue." These units and "standard specific activity" may be interconverted as follows:

$$\text{per cent dose per milligram element} = \frac{\text{standard specific activity}}{10}$$

$$\text{per cent dose per gram tissue} = \frac{\text{standard specific activity} \times \%\text{ dry matter} \times \text{milligram element/gram dry matter}}{1000}$$

of the plasma inorganic phosphorus and of the gastrointestinal tissues and contents.

The highest specific activity of the digesta is found in the small intestine (except in animal H-1, where the cecal contents have a slightly higher value). In the 8-months-old animals, 6 hours after injection of the tracer, the specific activity of the small intestinal contents is quite high as

TABLE 2

Standard specific activities of tissues and contents of the gastrointestinal tract of swine

$\mu c\ P^{32}/mg\ P$

mc P^{32} injected/kilogram body wt.

		2 MONTHS			4 MONTHS				8 MONTHS		
EXPERIMENT NO.		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
HRS. POST-INJECTION		5½	24	48	1	6	24	48	6	24	48
Plasma inorganic P		1.20	0.56	0.42	10.15	2.58	1.06	0.70	8.95	5.56	4.74
Stomach	Epithelium	0.29	0.37	0.41	0.38	0.51	0.54	0.37	0.49	1.23	0.61
	Contents	0.28	0.29	0.02					0.35	0.30	0.48
Small intestine	Tissue	0.52	0.64	0.50	0.61	0.55	0.60	0.46	1.16	1.71	1.08
	Contents	0.55	0.65	0.05					1.68	1.22	1.27
Cecum	Tissue	0.34	0.80	0.59	0.74	0.56	0.60	0.49	1.34	1.72	1.37
	Contents	0.59	0.38	0.05					1.11	0.74	0.91
Colon	Tissue	0.61	0.61	0.64	0.74	0.62	0.60	0.38	1.33	1.11	1.17
	Contents	0.23	0.26	0.05					0.02	0.64	0.87
Rectum	Tissue	0.49	0.57	0.48	0.63	0.57	0.53	0.39	1.17	1.10	1.26
	Contents	0.19	0.21	0.05					0.02	0.57	0.77

compared with other tissues, being exceeded only by liver and kidney (compare table 2 and Smith et al., '51).

In the 8-months-old animals during the first day after injection of the tracer the specific activity of the small intestinal contents is 4 to 5 times that of the stomach. The phosphorus concentration of the small intestinal contents is about 20% higher than that of the stomach contents ("fresh basis, calculated from table 1), and the capacity of the small

intestine is about 15% greater than that of the stomach. Thus, a much larger amount of radioactive (endogenous) phosphorus appears to enter the small intestine than the stomach.

Kjerulf-Jensen ('41–'42), using radiophosphate, has studied the secretion of endogenous phosphorus into the gastrointestinal tract of rats and into isolated intestinal loops. About 5 times as much endogenous phosphorus appeared in the jejunum as in the stomach, and very little appeared in the cecum 30 minutes after the administration of the tracer. Thus, it would appear that the main site of transfer of endogenous phosphorus into the digestive tract of animals with simple stomachs (viz., swine and rats) is the small intestine, and very little is secreted anterior to that portion.

As compared with the small intestinal contents, the contents of the digestive tract posterior to the small intestine show a decreased specific activity at all times after the administration of the tracer. This decrease can not result from a further secretion of endogenous phosphorus of low specific activity, since the plasma inorganic phosphorus, which is the precursor of endogenous phosphorus, is still quite highly labeled at the times involved (see table 2). Also, the low level of specific activity of the lower intestinal contents in the animals sacrificed soon after injection indicates only a small secretion in these regions. This reduction in specific activity of the digesta indicates a greater removal of radiophosphorus than of total phosphorus, which could result from a relatively greater resorption of endogenous (i.e., labeled) phosphorus than of food phosphorus in the lower portions of the digestive tract.

This apparent selective resorption of endogenous phosphorus may result from a functional deficiency of some digestive systems with simple stomachs, some dietary phosphorous compounds (viz., phytin) remaining undigested or otherwise unavailable. McCance and Widdowson ('35) have shown that a large part of the phytin ingested by man is excreted unchanged in the feces.

The specific activity relationships observed between the contents of various segments of the gastrointestinal tract of 8-months-old swine were also observed in the two-months-old swine. The levels of specific activity of the digesta, however, are much lower in comparable regions and at comparable times in the younger animals than in the older ones, except in the colon and rectum 6 hours after tracer administration. A similar situation has been reported regarding the secretion of endogenous calcium into the digestive tracts of young and mature rats (Wallace et al., '51). Six hours and later after intramuscular injection of radiocalcium, the percentage of the injected dose found in the digesta was much greater in the mature than in the younger animals. This difference in isotopic content of the digesta was interpreted as an indication of a greater re-absorption of calcium from the gut of the young animal.

It seems more likely, however, that the lesser quantity of isotope in the digesta of the young animal results from its lesser secretion. In young animals, bone formation removes larger amounts of some mineral elements from the circulating blood, which would lead to a more rapid rate of disappearance of an isotopic label, following a single tracer administration, than would be the case in mature animals. A more rapid rate of decrease of the injected tracer from the plasma inorganic phosphorus of young animals is evident in table 2, and the effect of this decrease on the specific activity of body tissues generally has been previously noted (Smith et al., '51).

When the specific activity relative to that of the plasma inorganic phosphorus ("relative specific activity"[6]) is calculated, the situation is reversed, the relative specific activities of the digesta 6 and 24 hours after injection in the two-months-old swine being much greater than those of the 8-months-old swine. The relative specific activities of colonic and rectal contents 6 hours after injection in the two-months-old swine are almost 100 times as high as the comparable material

[6] Relative specific activity $= \frac{\text{specific activity of tissue phosphorus}}{\text{specific activity of plasma inorganic phosphorus}}$

in the 8-months-old swine. This marked difference in specific activity is probably due to a greater intestinal motility in the younger animal labeled phosphorus secreted in the upper portions of the tract reaching the lower intestine sooner in the young animal than in the older.

At 48 hours after the injection the relative specific activites of digesta of the younger animals are much lower than those of the older animals, the values in the two-months-old animals being only 4 to 7% of those of the corresponding material in the older animals. This condition can also be explained on the basis of a greater intestinal motility in the younger animals, the older ones merely requiring a longer time to eliminate the endogenous (labeled) phosphorus as well as other materials from the digestive tract.

The distribution of the intravenously injected radiophosphate among the tissues of the gastrointestinal tract is somewhat different from that of the contents. At no time after tracer administration do the tissues of a particular animal vary as widely in radioactivity as do the contents. The maximum specific activity of the tissue is found somewhat lower along the digestive tract than is the case with the contents; it usually occurs in cecal or colonic tissue rather than in the small intestine. The tissue radioactivity does not merely reflect the passage of phosphorus between the blood and the contents, although this must be one factor in determining the tissue radioactivity. At early times after injection (viz., 6 hours in the mature animals) practically no radiophosphate is present in the colonic and rectal contents, indicating no secretion. At these times, however, the maximum specific activity of gastrointestinal tissue is generally found in the cecum, colon or rectum.

The metabolic activity of the tissue with respect to phosphorus can be evaluated by determining the rate with which it exchanges with the circulating inorganic phosphorus. For this purpose, the relative specific activities [7] of the tissues were calculated from the data given in table 2. The relative

[7] See footnote 6, p. 503.

specific activity represents the degree of equilibration of the tissue phosphorus with the plasma inorganic phosphorus, with respect to the tracer; the relative specific activity of the plasma inorganic phosphorus is always unity. In the youngest animals studied, the relative specific activity of all tissues except stomach exceeded unity within 24 hours after injection. On the basis of simple exchange (i.e., with no net loss or retention of phosphorus by the tissue), the specific activity of the plasma inorganic phosphorus should be limiting, and relative specific activities above unit would not be observed. The two-months-old animals used in this study, however, were growing, with an increase of 1.6% of their body weight per day (calculated from the body weights of the two- and 4-months-old groups). In the youngest animals, there was a net deposition of the tracer which accounted for the high relative specific activities of their tissues. The relative specific activity of the gastrointestinal tissues of the 4-months-old animals did not exceed a value of 0.7, and of the 8-months-old animals did not exceed 0.4.

SUMMARY AND CONCLUSIONS

The distribution in swine of intravenously injected radiophosphate at various times after its administration indicates a very large and rapid transfer of plasma inorganic phosphorus to the gastrointestinal contents and tissues. Most of this endogenous phosphorus appears to enter the contents of the small intestine, which, 6 hours after injection in the 8-months-old swine, has a specific activity greater than most tissues and organs, being exceeded only by the liver and kidney. A decreasing specific activity of the contents toward the posterior portion of the digestive tract indicates that the endogenous phosphorus is resorbed from the lower intestinal contents to a much greater extent than the food phosphorus. Ageing markedly decreases the secretion of endogenous phosphorus (as compared with the circulating inorganic phosphorus) and also decreases the rate of uptake

of circulating inorganic phosphorus by the gastrointestinal tissues.

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TRANSFER OF PHOSPHATE IN THE DIGESTIVE TRACT

II. SHEEP [1]

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In a previous report Smith et al. ('55) described the secretion of intravenously injected phosphate into the digestive tract of an animal species with a simple stomach, namely, swine. The digestive process of ruminants, such as sheep, is more complicated than that of nonruminants, principally because of prolonged storage of ingested material in the rumen and the action of microorganisms thereon. Ruminant digestion also involves an extensive secretion and resorption of minerals, which has been recognized since the early work of Wildt (1874, 1879). Using the silica of the feed as an inert "reference" substance, he was able to measure, in the digesta of sacrificed sheep, the secretion or absorption of several minerals that occurred in the various segments of the digestive tract. One of the elements principally involved in this exchange was phosphorus, of which 10 times the daily food intake was secreted into the various parts of the ruminant stomach.

Large quantities of low molecular weight (volatile) fatty acids are characteristically produced in the rumen (Elsden

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et al., '45–'46), and it has been suggested that the secretion of certain anions into the rumen serves to buffer them. Watson ('33) measured the pH of the ruminal contents of sheep and observed that it lies within the efficient buffering range of phosphate. Watson and others (Brünnich and Winks, '31; McDougall, '48) have proposed that secreted phosphorus enters the rumen via the saliva in the process of remastication and resalivation. Saliva phosphorus, which is almost entirely inorganic, is at least 4 times as concentrated in sheep saliva as in human beings and has about 15 times the concentration of inorganic phosphorus of the plasma.

METHOD

Sheep for this study were of three age groups: 1, 4, and 10 months, containing, respectively, 3, 6, and 6 animals. The mean body weights for these groups were: 10 ± 2 kg, 17 ± 2 kg, and 51 ± 4 kg, which would correspond respectively to 20, 50, and 90% of their mature weight. The one-month-old sheep were still nursing. A buffered isotopic solution containing radiophosphate was injected into the jugular vein of each. The animals were then returned to their usual routine until time of slaughter, which varied from one-half hour to 72 hours after injection. After sacrifice, samples of contents and tissue were collected from the following segments of the digestive tract: rumen, omasum, abomasum, small intestine (at the midpoint after the entry of the bile and pancreatic juice), cecum, colon and rectum. The epithelial lining was separated from the underlying muscle in the stomach tissues, and the rectal tissue was separated from the sphincter muscle prior to processing. The other tissues were merely freed of fat, fascia and intestinal contents and then processed as the entire tissue.

Determinations of dry matter, phosphorus and radioactivity were made on each sample. The experimental animals and details of the analytical procedures used have been previously described (Smith et al., '52).

RESULTS AND DISCUSSION

The results of the chemical analyses (percentage of dry matter, and milligrams of phosphorus per gram dry matter) of gastrointestinal contents and tissues are summarized in table 1 as the means and their standard deviations for the animals of each age group. As in the case of swine, some progressive changes in the composition of tissues and contents were observed along the tract. The decreased water content of the tissues posterior to the small intestine is similar to that observed in swine; however, the omasal tissue is generally the most hydrated. The decrease in phosphorus concentration in tissues posterior to the small intestine is also similar. The phosphorus concentration of the sheep gastrointestinal tissues is generally greater than that observed in swine, and this is also true of other organs and tissues of the two species (Smith et al., '52).

In the gastrointestinal contents, the highest water concentration occurs in the abomasum, except for the youngest lambs, which were on a liquid diet, and this decreases lower down. The dry matter results agree closely with data reported by Garton ('51) and Wildt (1874, 1879), but vary in some segments from the data of Brünnich and Winks ('31). The increasing phosphorus concentration in the contents of the gastrointestinal tract of swine was not evident in sheep. The highest phosphorus concentration was observed in the omasal contents, except in the one-month-old lambs, and from that point down it generally decreased. These results are essentially the same as reported by Brünnich and Winks ('31). Garton ('51) reports a similar relationship for the filtered liquors of the various parts of the ruminant stomach: ruminal liquor, 79 mg %; omasal liquor, 120 mg %; and abomasal liquor, 63 mg%. Sheep appear to absorb a much greater amount of the phosphorus in the digestive tract than swine. Although the phosphorus concentration in the 10-month-old sheep abomasal contents is 4 times that in the 8-month-old swine stomach (dry basis), the sheep rectal contents have only half the phosphorus concentration observed in swine.

TABLE 1

Dry matter and phosphorus concentration in tissues and contents of the gastrointestinal tract of sheep
(Mean and its standard deviation)

	1 MONTH		4 MONTHS		10 MONTHS		WILDT	BRUNNICH AND WINKS	GARTON
	Tissue	Contents	Tissue	Contents	Tissue	Contents	Contents	Contents	Contents
Dry matter, %									
Rumen	19.9 ± 1.7	12.2 ± 2.0	16.6 ± 1.3	10.6 ± 0.6	20.2 ± 1.1	11.5 ± 0.7	12.7 ± 1.6 [1]		11.4 ± 0.8 [2]
Omasum	16.9 [3]	36.1 ± 4.0	12.2 ± 1.2	16.3 ± 2.0	14.8 ± 0.5	18.8 ± 0.5	16.8 ± 0.2		19.2 ± 1.1
Abomasum	20.0 ± 1.3	20.4 ± 3.2	14.7 ± 0.8	6.5 ± 1.6	20.3 ± 0.5	7.3 ± 1.2	8.5 ± 0.9 [4]		8.0 ± 0.9
Small intestine	16.3 ± 0.8	13.1 ± 2.7	15.5 ± 1.6	7.2 ± 1.0	16.4 ± 0.4	8.7 ± 0.6	8.0 ± 1.0		
Cecum	18.7 ± 1.8	19.3 ± 2.3	15.1 ± 0.7	8.9 ± 1.6	17.9 ± 0.3	11.8 ± 0.7	12.3 ± 0.2		
Colon	22.3 ± 2.1	27.9 ± 1.8	15.5 ± 1.5	11.3 ± 1.9	23.8 ± 1.5	17.8 ± 1.1	15.3 ± 0.3		
Rectum	25.7 ± 3.3	49.0 ± 1.8	18.6 ± 1.5	15.2 ± 2.7	25.5 ± 2.1	33.5 ± 2.7	25.9 ± 1.6		
P content, mg P/gm dry weight									
Rumen	11.9 ± 1.8	21.2 ± 2.7	22.8 ± 4.0	8.0 ± 0.8	10.7 ± 1.5	10.5 ± 0.7	6.5 ± 0.4	9.4 ± 1.8 [5]	
Omasum	11.3 ± 0.7	6.2 ± 0.8	10.7 ± 0.5	16.3 ± 2.3	8.2 ± 0.4	13.4 ± 0.7	8.1 ± 0.9	14.2 ± 3.3	
Abomasum	11.4 ± 1.4	6.1 ± 0.7	14.9 ± 0.4	11.1 ± 1.1	9.9 ± 0.7	12.6 ± 1.4	8.4 ± 0.6	13.2 ± 0.3	
Small intestine	18.3 ± 2.4	7.4 ± 1.2	17.3 ± 1.4	13.2 ± 0.6	13.3 ± 0.5	12.3 ± 0.6	8.0 ± 0.8	10.3 ± 1.3	
Cecum	12.8 ± 0.4	6.4 ± 1.2	12.7 ± 0.9	9.5 ± 1.0	8.5 ± 0.3	6.6 ± 0.6	5.3 ± 0.7	7.9 ± 1.6	
Colon	11.7 ± 0.5	6.4 ± 1.2	11.9 ± 0.9	11.9 ± 1.6	8.8 ± 0.4	6.6 ± 0.3	4 9 ± 0.8		
Rectum	9.5 ± 1.1	7.2 ± 1.5	8.6 ± 0.3	9.9 ± 0.8	5.5 ± 0.4	7.3 ± 0.7	4.4 ± 0.9	7.1 ± 0.4	

[1] Mean of 5 sheep; calculated from Wildt's data.
[2] Mean of 7 sheep; calculated from Garton's data.
[3] One determination only.
[4] Included duodenum contents.
[5] Calculated from data for two healthy sheep of which all digesta were analyzed; phosphorus concentration in ruminal contents of 9 sheep, some of which were range animals, was: 8.6 ± 1.1.

The aging effects observed in the gastrointestinal tissues of swine, a decrease in hydration and phosphorus concentration, were not evident in these sheep.

The results of the chemical and isotopic analyses and the tracer dose per kilogram of body weight have been used to calculate the "standard specific activities" of the various samples, which are presented in table 2. The pattern of radiophosphate secretion into the gastrointestinal contents is quite different from that previously observed in swine, which was characterized by a maximum transfer of the tracer into the small intestinal or cecal contents with the radioactivity diminishing sharply in either direction along the tract. In the young nursing lambs the amount of radiophosphate entering the digestive tract is small as compared with the older sheep, and the maximum specific activity generally occurs in the ruminal contents. In the older sheep the maximum specific activity was generally found in the omasal or abomasal contents. These shifts in the site of major radiophosphate secretion into the gut contents indicate a change in the nature of the digestive process following weaning, involving a difference in the quantity or composition of the digestive secretions. McDougall ('48), however, found no effect of age on the composition of sheep saliva.

This secretion of phosphate into the contents of the digestive tract is much more extensive in sheep than in swine, the maximum standard specific activities observed in sheep being about twice as great as those observed in swine. The dietary intake of the two species is the reverse, the phosphorus requirement per unit of body weight being about twice as great for swine as for sheep (Albritton, '53). When these specific activities are compared with the specific activity of the plasma inorganic phosphorus (i.e., "relative specific activity"[4]), the species difference is more pronounced. In mature sheep the relative specific activity of the gastrointestinal contents is approximately unity within 24 hours after

[4] Relative specific activity $= \frac{\text{Specific activity of tissue phosphorus}}{\text{Specific activity of plasma inorganic phosphorus}}$

TABLE 2

Standard specific activity of tissues and contents of the gastrointestinal tract of sheep

Results expressed as: $\frac{\mu c\ P^{32}/mg\ P}{mc\ P^{32}\ injected/kilogram\ body\ weight}$

		1 MONTH OLD			4 MONTHS OLD					10 MONTHS OLD					
Experiment no.		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Hrs. post-injection		1	6	24	½	1	3	6	24	48	1	4	8	24	48	72
Body weight, kg		10.7	12.7	6.6	14.8	24.1	15.0	16.4	18.2	13.9	45.5	72.7	46.5	45.3	51.4	53.6
Plasma inorg. P		2.88	0.95	0.31	15.68	16.02	3.69	1.94	0.86	0.71	17.68	4.78	2.27	1.18	0.76	0.63
Rumen	Epithelium	0.25	0.47	0.26	0.36	0.56	0.37	0.67	0.95	0.63	0.38	1.09	0.86	1.19	0.84	0.61
	Contents	0.16	0.27	0.35	0.38	0.54	0.42	1.17	0.96	0.62	0.05	1.89	1.60	1.19	0.72	0.73
Omasum	Epithelium	0.12	0.51	0.27	0.44	0.55	0.94	1.27	0.77	0.68	0.75	1.46	1.02	0.94	0.77	0.75
	Contents	0.04	0.05	0.41	...	...	0.92	2.30	...	...	0.58	1.80	1.36	1.26	0.72	0.70
Abomasum	Epithelium	0.11	0.12	0.12	0.22	0.26	0.38	0.80	0.47	0.59	0.32	0.46	0.46	0.49	0.60	0.66
	Contents	0.13	0.11	0.15	0.09	0.56	0.98	2.29	0.98	0.65	0.41	2.56	1.86	1.25	0.71	0.63
Small int.	Tissue	0.30	0.47	0.63	0.39	0.37	0.59	1.13	0.72	0.73	0.65	0.75	0.76	0.78	0.76	0.65
	Contents	...	0.14	0.24	0.31	0.33	0.38	1.36	0.93	0.99	0.48	0.53	1.20	1.11	0.87	0.82
Cæcum	Tissue	0.37	0.22	0.54	0.43	0.51	0.75	0.88	0.67	0.86	0.76	0.94	0.83	0.65	0.80	0.67
	Contents	0.01	0.05	0.27	0.04	0.13	0.08	0.12	1.00	0.86	0.16	0.15	0.34	1.26	0.75	0.82
Colon	Tissue	0.19	0.41	0.55	0.41	0.45	0.75	0.75	0.77	0.73	1.06	0.92	0.98	0.68	0.94	0.63
	Contents	0.03	0.03	0.29	0.10	0.19	0.11	0.13	1.04	0.87	0.21	0.10	0.53	1.30	0.63	0.80
Rectum	Tissue	0.34	0.31	0.23	0.51	0.48	0.79	0.72	0.55	0.71	0.98	1.09	0.83	0.56	0.68	0.57
	Contents	0.01	0.01	0.28	0.20	0.17	0.06	0.02	1.03	...	0.02	0.01	0.01	1.36	0.72	0.87

tracer injection whereas in the older swine the maximum relative specific activity at this time was less than 0.25, and this was not greatly increased 48 hours after injection.

In sheep the specific activities of the gastrointestinal contents soon after tracer administration indicate that the major part of the endogenous phosphorus is secreted into the various segments of the ruminant stomach. The secretion of radioactive phosphorus into the rumen, which can be used to estimate the rate of endogenous phosphorus secretion, can be calculated as: P^{32} secreted = P^{32} in rumen + P^{32} lost from rumen.

The amount of P^{32} secreted into the rumen is determined by the secretion rate of phosphorus, S_R (grams phosphorus per hour), and its specific activity during the period of secretion. It has been shown that the specific activity of phosphorus in the digestive secretions following injection of radioactive phosphate is the same as that for the plasma inorganic phosphorus (Kjerulf-Jensen, '41–'42). Consequently, the specific activity of endogenous phosphorus can be represented as the specific activity of plasma inorganic phosphorus, π:

$$P^{32} \text{ secreted} = S_R \int_0^t \pi \, dt.$$

The amount of P^{32} in the rumen at any given time, t, is the product of the quantity of phosphorus in the rumen, R (grams of phosphorus), and its specific activity at that time $\rho_{(t)}$:

$$P^{32} \text{ in rumen} = R\,\rho_{(t)}.$$

The amount of P^{32} that is lost from the rumen is determined by the quantity of phosphorus leaving the rumen and the specific activity of ruminal phosphorus, ρ, during the period of loss. Assuming that the quantity of ruminal phosphorus, R (grams of phosphorus), is constant, the amount of phosphorus leaving the rumen will be equal to the sum of the dietary intake, D (grams of phosphorus per hour),

and the endogenous secretion, S_R (grams of phosphorus per hour):

$$P^{32} \text{ lost from rumen} = (D + S_R) \int_0^t \rho \, dt.$$

By combining the last three expressions into the original equation, the secretion of P^{32} can be represented as:

$$S_R \int_0^t \pi \, dt = R\rho_{(t)} + (D + S_R) \int_0^t \rho \, dt,$$

which can be rearranged to calculate the rate of secretion of phosphorus in the rumen, S_R (grams of phosphorus per hour):

$$S_R = \frac{R\rho_{(t)} + D\int_0^t \rho \, dt}{\int_0^t \pi \, dt - \int_0^t \rho \, dt}$$

The quantity of phosphorus in the rumen, R, was calculated from the chemical composition of ruminal contents (table 1) and the quantity of rumen contents taken from the data of Washburn and Brody ('37, table 8).[5] Since the rumens of the 4-month-old sheep appeared to be functional, it was assumed that their contents had the same proportionality to body size, i.e., 15% of live weight, as those of the 10-month-

[5] There is some disagreement among the several statements of the quantity and distribution of the "fill" in sheep, particularly concerning the ruminal and omasal contents. We have used the data of Washburn and Brody ('37) in our calculations since they were derived from a large number of animals of about the same body size as those reported herein. Following is a summary of the gastrointestinal contents, relative to live weight, which was calculated from the indicated reports:

	GASTROINTESTINAL CONTENT, % OF LIVE WEIGHT		
	Wildt (1874, 1879)	Washburn and Brody ('37)	Elsden et al. ('45–'46)
No. sheep	5	13	3
Body weight (kg)	36.0 ± 2.7	53.6 ± 1.7	82.0 ± 2. 7
Rumen and reticulum	11.4 ± 0.6%	15.0 ± 0.9%	8.8 ± 1. 0%
Omasum	0.6 ± 0.1%	0.5 ± 0.1%	0.1 ± 0.04%
Abomasum [1]	0.9 ± 0.1%	1.1 ± 0.1%	0.8 ± 0. 1%
Small intestine	2.2 ± 0.5%	1.5 ± 0.1%	1.0 ± 0. 3%
Large intestine	2.8 ± 0.2%	2.5 ± 0.2%	1.5 ± 0. 2%

[1] Includes duodenum.

old sheep. Accordingly, the rumens of the 4-month-old sheep contained 2.16 gm of phosphorus, and of the 10-month-old sheep, 8.65 gm of phosphorus. The dietary phosphorus intake, D, was estimated from Albritton's ('53) data as 90 and 45 mg of phosphorus per kilogram of body weight per day for the 10- and 4-month-old sheep respectively. The specific activity of the ruminal phosphorus at specific times, $\rho_{(t)}$, is given in table 2. The specific activity of the ruminal phosphorus over various periods following tracer administration, $\int_o^t \rho \, dt$, was calculated from the mean specific activity and time of each period. The specific activity of the plasma inorganic phosphorus over various intervals after tracer injection, $\int_o^t \pi \, dt$, was similarly calculated.[6] The results of these calculations are summarized in table 3 as the mean secretion rates, and their standard errors, for the 4- and 10-month-old sheep. In this calculation, as well as those for other parts of the ruminant stomach, the derived rate for the earliest period was quite small, and that for three to 4 hours after injection was rather large. This suggests some delay in transfer of circu-

[6] The standard specific activity of the plasma inorganic phosphorus over a period of time following the injection of the tracer was calculated by plotting its standard specific activities as a function of time and integrating the area under the curve. In addition to the observed values, the standard specific activity at "zero time" was calculated, and several intermediate values were semi-logarithmically interpolated.

The standard specific activity at "zero time" was calculated by:

(a) Assuming that the tracer was distributed only through the plasma and interstitial fluid inorganic phosphorus, both of which are in rapid exchange. The plasma volume was assumed to be 50 ml per kilogram, and the interstitial fluid, 250 ml per kilogram of body weight (cf., Kleiber et al., '50).

(b) Assuming that the inorganic phosphorus concentration was the same for both fluids and equal to that measured in the plasma; 8.0 mg % for the 10-month-old sheep, and 10 mg% for the 4-month-old sheep.

(c) Thus, the original dilution of the tracer was through 1225 mg phosphorus in the 10-month-old sheep (body wt. = 51 kg) and through 480 mg phosphorus for the 4-month-old sheep (body wt. = 17 kg). The standard specific activity of the plasma inorganic phosphorus at "zero time" after the injection of "X" mc of labeled phosphate would be:

$$\text{Standard specific activity} = \frac{\mu c\ P^{32}/mg\ P}{mc\ P^{32}\ \text{injected/kilogram body wt.}}$$

$$= \frac{1000\ \text{"x"}/1225}{\text{"x"}/51} = 42 \text{ in 10-month-old sheep}$$

$$= \frac{1000\ \text{"x"}/480}{\text{"x"}/17} = 33 \text{ in 4-month-old sheep}$$

lating phosphate. Consequently, the mean value for all animals was used, which should minimize the effect of such a delay.

The mean ruminal secretion of phosphorus in the 6 10-month-old sheep is about 0.20 gm per hour, or 4.8 gm per day (the major part of the salivary secretion is continuous; McAnally and Phillipson, '44). If saliva, which has a phosphorus concentration of 0.81 gm per liter (McDougall, '48), were the only source of this secreted phosphorus, it would have to flow at the rate of 6.0 liters per day. This salivation rate is greater than Watson's estimate ('33) of 4 to 5 liters per day, which was based on the excess of ruminal phosphorus over that ingested and would thus include all endogenous phosphorus as salivary phosphorus. The saliva production rate, calculated herein, is much larger than earlier estimates (see Watson, '33) of one to two liters per day, which presumably were measurements of direct flow. Thus, a large part, perhaps half, of the endogenous phosphorus appears to enter the rumen from some source other than saliva, and it probably enters directly through the rumen wall since the ruminal epithelium is devoid of secretory glands. It has been shown by Parthasarathy et al. ('52) that the rumen wall *in vivo* is permeable to phosphate in either direction. A histological examination of the walls of the ruminant stomach by Barcroft et al. ('44a) shows that they are quite vascular and apparently well suited to exchange. If as little as a third of the endogenous phosphorus entering the rumen of the 10-month-old sheep (i.e., 1.6 gm of the 4.8 gm of phosphorus per day) were to enter through the rumen wall and at the same concentration as the plasma inorganic phosphorus (i.e., 8 mg % of phosphorus), this would require a fluid transfer of 20 liters per day, which is greatly in excess of the saliva flow.

The endogenous phosphorus secretion reported herein, 4.8 gm per day, is larger than the 3.5 gm per day reported by Wildt (1874, 1879) but is about the same when expressed per kilogram of body weight. However, his sheep were on a much

TABLE 3

Endogenous phosphorus secretion in sheep

TYPE OF SECRETION	4 MONTHS OLD		10 MONTHS OLD	
	Per animal	Per kg [1] body wt.	Per animal	Per kg [1] body wt.
Rumen secretion (S_R), mg P/hr.	68 ± 8	4.0	198 ± 41	3.9
Omasum secretion (S_O), mg P/hr.	16 ± 9 [2]	0.9 [2]	13 ± 3	0.3
Abomasum secretion (S_A), mg P/hr.	9 ± 3 [2]	0.5 [2]	35 ± 6	0.7
Total endogenous phosphorus ($S_R + S_O + S_A$) mg P/hr.	98 ± 19	5.8	246 ± 46	4.8
Rumen secretion per gram dietary phosphorus (S_R/D), mg P/gm dietary P	1.1		2.1	
Rumen secretion per gram rumen phosphorus (S_R/R), mg P/gm rumen P/hr.	31.5		24.3	
Rumen secretion, per cent of total endogenous secretion $[(S_R/S_R + S_O + S_A) \times 100]$, %	90.0		80.0	
Rumen secretion per kilogram dry feed consumed, gm P/kg feed	2.3		3.2	
Turnover time of rumen phosphorus ($R/S_R + D$), hrs.	16.4		27.7	

[1] Body weight of 4-month-old sheep = 17 ± 1 kg; of 10-month-old = 51 ± 4 kg.
[2] Two animals only.

lower dietary level (0.012 gm per kilogram of body weight per day) than that used in these calculations (0.045 gm per kilogram of body weight per day).[7] Thus, Wildt found an endogenous secretion of phosphorus into the rumen which was 8.3 times as large as the daily intake, whereas our calculations indicate a ratio of 2.6.

The ruminal phosphorus secretion was also calculated for the 4-month-old sheep (table 3) and it had a mean, for the 6 animals, of 0 07 gm per hour. The ruminal secretion in the two age groups appears to be the same per unit of body size, but is somewhat different in relation to other nutritional or physiological factors (table 3). The younger animals appear to secrete less phosphorus in the rumen as compared with the dietary phosphorus or dry feed intake (the 4-month-old sheep were assumed to ingest 1.7 gm dry feed per kilogram per hour, and the 10-month-old sheep, 1.2 gm feed per kilogram per hour; Albritton, '53). The younger sheep appear to secrete more phosphorus into the rumen as compared with the total endogenous secretion, or with the total quantity of phosphorus in the rumen. The rumen phosphorus seems much more mobile in the younger animals, having a much shorter "turnover time."

The high radioactivity observed in the omasal contents, which is particularly apparent in the 4- and 10-month-old sheep one hour after injection, suggests a secretion of endogenous phosphorus in that organ. It is possible that this organ might receive saliva directly via the esophageal groove and hence develop a higher specific activity than the ruminal contents without local secretion. However, the high concentration of phosphorus in the omasal liquor, which exceeds that of ruminal liquor (Garton, '51) or that reported for

[7] In order to increase the sensitivity of his method, Wildt selected feeds which were quite low in nutritive materials. With his first two sheep (Wildt, 1874) he used meadow hay which contained 0.27% phosphorus, and with his other three sheep (Wildt, 1879) he used barley straw which contained 0.08% phosphorus. The animals were fed this ration for a 10-day preliminary period, which apparently did not affect the secretion rate of endogenous phosphorus (viz., the data on ruminal secretion from his two experiments were essentially the same, and also equivalent, per kilogram of body weight, to that reported herein).

saliva (Watson, '33; McDougal, '48), indicates the presence of an active process, either phosphorus secretion or selective water resorption. Singleton ('53) notes that the epithelium of this organ is permeable to water and suggests that the dehydration of the contents is more from water absorption than from a wringing-out of the fluid from the digesta. From the study of Barcroft, McAnally and Phillipson ('44a) it appears that the folds of the omasum are the most vascularized of the epithelia of the ruminant stomach. It is also known (Barcroft et al., '44b) that a significant absorption of volatile fatty acids occurs in this organ, to the extent that digesta entering the abomasum are essentially free of these materials.

The rate of secretion of phosphorus into the abomasum can be calculated from the specific activity of the contents, as was previously done for the rumen. In this case, some of the P^{32} entering the omasum is from the passage of materials along the digestive tract, rather than from the secretion alone, as in the rumen:

P^{32} secreted = P^{32} in omasum + P^{32} lost from the omasum — P^{32} entering from rumen.

The phosphorus entering the omasum can be considered as that from the diet and from secretion into the rumen, since absorption of phosphorus in the ruminant stomach is quite small (Singleton, '53; Scarisbrick and Ewer, '51). The phosphorus leaving the omasum is considered to be the sum of that entering and that secreted therein. Thus, the P^{32} secreted in the omasum can be represented as:

$$S_O \int_0^t \pi \, dt = O_{o(t)} + (D + S_R + S_O) \int_0^t o \, dt - (D + S_R) \int_0^t \rho \, dt,$$

which can be rearranged to calculate the rate of secretion of phosphorus in the omasum, S_O (grams per hour):

$$S_O = \frac{O_{o(t)} + (D + S_R)\left(\int_0^t o \, dt - \int_0^t \rho \, dt\right)}{\int_0^t \pi \, dt - \int_0^t o \, dt}$$

Where: O is omasum phosphorus in grams
o is the omasum phosphorus specific activity (at time, $t = o_{(t)}$)
S is the secretion rate (grams of phosphorus per hour): in the rumen, S_R, and in the omasum S_O
D is the dietary intake of phosphorus (grams of phosphorus per hour)
ρ is the specific activity of rumen contents, and
π is the specific activity of plasma inorganic phosphorus.

The quantity of omasum phosphorus, O, was calculated from the composition of omasum contents (table 1) and the quantity of omasum contents, which was taken from the data of Washburn and Brody ('37).[8] Accordingly, the omasum of the 4-month-old animals should contain 0.23 gm of phosphorus, and of the 10-month-old animals, 0.63 gm of phosphorus. The specific activity of the omasum contents, $_{(t)}$, given in table 2, was integrated as previously indicated. Other values are the same as previously used in the calculations for the rumen.

The secretion of phosphorus in the omasum for all 6 mature sheep (see table 3) has a mean value of 0.013 gm per hour. This secretion is rather small when compared with the total quantity of phosphorus in the omasum contents, the hourly addition being only 2% of the phosphorus contained. It is also only a minor source of omasum phosphorus, amounting to 4 to 5% of the phosphorus entering the organ.

Wildt (1874, 1879) noted a marked decrease in phosphorus concentration, relative to the silica concentration, in the omasal contents, suggesting that this organ was a site of phosphorus absorption. The structure of the omasum suggests that siliceous particles might tend to be retained there, which would lead to artificial results by Wildt's method.

The tracer indication of secretory activity in the abomasum is expected, since this organ is the glandular portion of the

[8] See footnote 5, page 514.

ruminant stomach, and it is likely that circulating phosphorus would enter the digesta in this organ via the gastric juice. The rate of secretion of phosphorus in the abomasum can be calculated, as previously done for rumen and omasum, from the appearance of P^{32}:

P^{32} secreted = P^{32} in abomasum + P^{32} lost from abomasum — P^{32} entering from omasum.

By expressing P^{32} as phosphorus secreted and its specific activity, the following equation is obtained:

$$S_A \int_0^t \pi \, dt = A\,\alpha_{(t)} + (S_A + S_O + D)\int_0^t \alpha \, dt - (S_R + S_O + D)\int_0^t o \, dt,$$

which can be rearranged to calculate the rate of secretion of endogenous phosphorus in the abomasum, S_A (grams of phosphorus per hour):

$$S_A = \frac{A\,\alpha_{(t)} + (S_O + S_R + D)\left(\int_0^t \alpha \, dt - \int_0^t o \, dt\right)}{\int_0^t \pi \, dt - \int_0^t \alpha \, dt}$$

Where: A is the abomasal phosphorus content (gm)
α is the abomasal phosphorus specific activity (at time, $t = \alpha_{(t)}$), and other symbols have the same significance as previously indicated.

The quantity of abomasal phosphorus, A, was calculated from the composition indicated in table 1 and the quantity of abomasal contents.[9] Accordingly the abomasum of the 4-month-old sheep would contain 0.14 gm of phosphorus, and of the 10-month-old sheep, 0.52 gm of phosphorus. The specific activity of the abomasal contents, α, given in table 2 was integrated as previously indicated. Other values used are the same as for the previous calculations.

The secretion of phosphorus in the abomasum of the 10-month-old sheep (see table 3) has a mean value for all 6 animals of 0.035 gm per hour. If this secretion were continual, it would amount to 0.84 gm per day. This is close to

[9] See footnote 5, page 514.

the phosphorus secretion of 1.1 gm per day measured by Wildt (1874, 1879), but is relatively less when compared to the dietary intake. This secretion of phosphorus into the abomasum is small as compared with the total phosphorus in the abomasal contents, the hourly addition being about 7% of the phosphorus contained. It does, however, amount to more than 11% of the phosphorus entering the organ.

Very little radioactivity was observed in the intestinal contents until 24 hours after the injection, which was quite different from the observations in swine. The intestines seem to be of much less importance in sheep, in which they constitute less than 35% of the capacity of the gastrointestinal tract, than in swine, in which they constitute over 70% of the tract's capacity (Dukes, '47). Although there is a measurable radioactivity in the small intestinal, cecal and colonic contents one hour after injection, indicating some secretion or exchange at all points along the tract, this is small compared to anterior portions of the tract, and the small intestinal contents of the 10-month-old sheep are less radioactive than those of the 8-month-old swine at all times after the tracer injection. No great activity is evident in the intestinal contents until 6 or 8 hours after injection, and this seems due to a movement of digesta from the stomach portions. At 24 hours and later after injection, the specific activity of the gastrointestinal contents is essentially uniform. This indicates that the resorption of endogenous phosphorus is proportionately equal to the absorption of the exogenous phosphorus, which is contrary to the situation previously observed in swine.

In the 4- and 10-month-old sheep, where several rectal contents samples were collected soon after injection, there is evidence of a small but active secretion of endogenous phosphorus in that organ. Samples taken at one-half and at one hour after injection have a fairly high specific activity which decreases for 6 or 8 hours, as nonlabeled digesta move through this region and the specific activity of the blood decreases. Evidence for a phosphorus excretion in the rec-

tum is borne out by the data of Brünnich and Winks ('31), which show a 30 to 40% higher phosphorus concentration (per gram of dry matter) in the feces than in the rectal contents, but not by Wildt's data (1879) where the results were variable.

This internal circulation of minerals exists for other elements, however, with different levels and at different secretory sites. Wildt's data (1879) indicate that only a sodium secretion is associated with phosphorus secretion. Watson ('33) has noted a high salivary bicarbonate level, which would also share secretory sites with phosphate. Some ions, such as potassium and chloride, are secreted mainly in the abomasum, and others, such as calcium, do not enter the digesta until the small intestine (beyond the point of entry of bile and pancreatic juice). Recent tracer work with intravenously injected radiocobalt (Co^{60}) by Keener et al. ('51) has demonstrated that this element is deposited almost exclusively in the intestinal tract, particularly in the small intestine.

The appearance of the radioactive phosphorus in the tissues of the digestive tract followed a markedly different pattern from that observed in the contents. Whereas the abomasal contents generally had the highest specific activity observed along the tract up to 24 hours after injection, the abomasal tissue generally had the lowest specific activity. The lower intestinal tissues, unlike the contents, early acquired a specific activity comparable to that of the anterior gastrointestinal tissues. The colonic and rectal tissues did not reflect the observed early secretion of the tracer into the contents with a decrease in specific activity. Generally, there was no strict parallelism between the distribution of the radiophosphorus in the contents and tissues of the digestive tract, and it appeared that the phosphorus metabolism of the tissue is quite different from the phosphorus exchange between the gastrointestinal contents and the blood although tissue phosphorus must be intermediate in that exchange.

The phosphorus metabolism of the gastrointestinal tissues can be evaluated by measuring the rate at which the specific activity of the tissue phosphorus would equilibrate with the specific activity of the plasma inorganic phosphorus, the ultimate precursor of the tissue phosphorus. For this purpose, the "relative specific activities" (i.e., the ratio of the specific activities of tissue and plasma inorganic phosphorus) for the various gastrointestinal tissues were calculated from the data in table 2.

In the young nursing lambs, the specific activity of some tissues exceeded, almost doubling, that of the plasma inorganic phosphorus, indicating the growth phenomenon previously noted in young swine. In the older sheep, however, the specific activities of the phosphate in tissues and plasma were essentially equal 48 hours after injection of the tracer, and no consistent change was observed at 72 hours.

When the proportionate differences between the specific activities of tissue phosphorus and plasma inorganic phosphorus (i.e., 1-relative specific activity) are examined, they are found to be semi-logarithmically linear with time, at least up to 24 hours after injection. This indicates that the rate of uptake of the tracer by the tissue is proportional to the difference in specific activity between it and plasma phosphate, i.e., a "first order" process, and is probably a matter of simple exchange in this period soon after the injection. Processes of this order follow the equation:

$$1 - \text{relative specific activity} = be^{-kt}$$

where: b is the difference existing at the time of injection and is unity in this case, since at that time none of the tracer had been added to the tissue.

$-k$ is the rate constant for the equilibration of the two specific activities, i.e., for the rate of change of the quantity (1-relative specific activity). It is negative to indicate a decrease in the specific activity difference with increasing time.

The equilibration rate constants, indicating the phosphorus metabolic activity of the tissues, have been calculated and are presented in table 4. It appears that with increasing age

most of the tissues exhibit a decreasing ability to take up (exchange) plasma phosphate, the possible exceptions being those of the abomasum and rectum. This aging effect is particularly marked in the rumen and small intestine.

TABLE 4

Equilibration rate of gastrointestinal tissue phosphorus and plasma phosphate in sheep of different ages

(1-relative specific activity = be^{-kt})

TISSUE	$k \times 100$ [1]		
	1 MONTH	4 MONTHS	10 MONTHS
Rumen	— 9.0	— 6.8	— 5.8
Omasum	— 10.3	— 9.6	— 7.0
Abomasum	— 2.0	— 3.4	— 3.3
Small intestine	— 12.1	— 7.7	— 4.7
Cecum	— 6.4	— 6.2	— 5.4
Colon	..	— 7.7	— 5.5
Rectum	— 6.4	— 5.8	— 6.0

[1] Since t is measured in hours, $k \times 100$ is the percentage change in the specific activity difference per hour. Assuming that only a simple exchange exists between tissue and circulating phosphate, this would represent the percentage of tissue phosphorus replaced by plasma inorganic phosphate per hour.

SUMMARY AND CONCLUSIONS

The distribution of intravenously injected radiophosphorus among the contents of the sheep digestive tract indicates a mineral exchange between these materials and the blood. This exchange for phosphorus was more active than that previously observed in swine and involved different secretory sites. Phosphate, which may function as a buffer for volatile fatty acids produced in the rumen, enters the rumen via the saliva, and in apparently even larger quantities directly through the rumen wall. A rapid increase in radioactivity of the omasal contents indicates phosphorus secretion in this organ. The principal site of endogenous phosphorus secretion seems to be the ruminant stomach, and very little phosphorus enters the intestines. An early appearance of radioactivity of the rectal contents indicates a small but active secretion of phosphorus in this region.

A pronounced aging effect is noted in the phosphorus exchange of the gastrointestinal tissues of sheep. Aging in sheep, however, does not lead to change in chemical composition of the body as it does in swine.

ACKNOWLEDGMENT

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RELATIONSHIP OF DIETARY PROTEIN AND FOOD INTAKE TO PYRIDOXINE NUTRITION IN THE RAT[1]

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FOUR FIGURES

(Received for publication July 21, 1955)

The major components of the diet greatly influence the requirements for certain water-soluble vitamins in animals. The concentrations of riboflavin, niacin (Sarett and Perlzweig, '43) and pyridoxine (Sheppard and McHenry, '46) in rat liver have been reported to vary directly with the protein level of the diet. On the other hand, the concentrations of thiamine in the carcass and liver mainly reflect the intake of the vitamin rather than intake of protein (Sarett and Perlzweig, '43). Seifter et al. ('48) found that riboflavin and niacin were lost from the liver at a faster rate than nitrogen in animals fed non-protein diets. In studies with high protein diets, a greater demand for pyridoxine occurs (Foy and Cerecedo, '41). This effect has been attributed mainly to increased levels of amino acids, such as tryptophan and methionine, whose utilization specifically requires pyridoxine (Miller and Baumann, '45; De Bey et al., '52).

From numerous studies it has been observed that the level of protein in the diet markedly influences the activity of

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certain tissue enzymes, particularly xanthine oxidase, which is known to require riboflavin as a part of the coenzyme (Williams et al., '50; Litwack et al., '50, '52, '53a, b, '54). These effects are usually attributed mainly to loss in apoenzyme. Under certain conditions, however, the loss in enzyme activity appears to be due to the inability of animals to synthesize or maintain the proper metabolism of certain vitamin-containing coenzymes [3] (Arata et al., '55). A comparison of the effects of inanition (Miller, '48) with low- or non-protein diets indicates that inanition generally produces less decrease in the activity of liver enzymes than the use of diets low or lacking in protein.

Throughout the world, human dietary habits are found which roughly correspond to the different feeding procedures discussed above: (1) Restricted protein intake, (2) high protein intake, and (3) restricted food intake. If, in addition to these abnormal dietary patterns, one or more vitamins is borderline or deficient, the storage and utilization of the vitamin in question could be profoundly influenced.

The purpose of the present paper is to present results of some of our studies on the relationship of protein and food intake to storage of tissue vitamins, and thus to development of vitamin deficiencies. The first vitamin chosen for these studies is pyridoxine because of its central role in protein and amino acid metabolism. In this work we have investigated the effects of the dietary patterns listed above on the development of a pyridoxine deficiency as measured by depletion of the vitamin from the tissues of albino rats. To observe the effects of dietary history on vitamin repletion, we have also studied the restoration of tissue pyridoxine in rats which had previously received the various dietary regimens deficient in pyridoxine.

EXPERIMENTAL

Male albino rats of the Holtzman strain were used as experimental animals. Fifty-six rats, each weighing approxi-

[3] Unpublished results from this laboratory.

mately 75 gm, were separated into 5 groups of 8 to 12 rats each. Group I (*positive control*) consisting of 8 rats, received a ration containing, in per cent, vitamin-test casein,[4] 20; Salts IV,[5] 4; corn oil, 5; vitamin mix[6] plus 1 μg of vitamin B_{12} per gram of vitamin mix, 2; and sucrose, 69. Group II (*negative control,* 12 rats) received the same diet as group I except that pyridoxine was omitted from the vitamin mix. Group III (*restricted food intake,* 12 rats) was fed the same diet as group II but was allowed only one-third of the quantity of ration consumed by the rats in group I for the first 4 weeks. Group IV (*high protein,* 12 rats) differed from group II in that the casein in the ration was increased to 60% at the expense of sucrose. In group V (*non-protein,* 12 rats) casein was completely omitted from the diet and replaced by sucrose. Vitamins A, D, E and K in 90% ethanol were administered orally to the rats once each week to supply at least twice the minimum requirement of the rat.

The animals were housed individually in raised-bottom cages and were weighed once a week. After one month, 4 rats from each of the groups were sacrificed; their livers, kidneys and intestines were removed, weighed and kept frozen at −4°C. until vitamin B_6 activity was estimated. The small intestine (duodenum to the termination of the ileum) was cut open and washed thoroughly with cold water before blotting, weighing, and storing. To study the specificity of the various effects on pyridoxine, riboflavin estimations were also carried out on all of these organ samples.

Next, the effects of the previous feeding of the various diets listed above on pyridoxine repletion were studied. After the initial 4-week period, the remaining 8 rats of groups II to V were divided into two sub-groups of 4 rats each (groups IIA, IIB to VA and VB). Groups IIA to VA were fed the 20% casein, pyridoxine-deficient ration; the others (groups IIB to VB) were fed the same ration supplemented with

[4] General Biochemicals Company, Inc.
[5] Hegsted et al. ('41).
[6] Williams and Elvehjem ('49).

0.25 mg pyridoxine per 100 gm ration. All of the rats were fed these rations ad libitum for two weeks after the initial 4-week depletion period. The livers, kidneys and intestines were then removed and treated as outlined above. Vitamin B_6 activity was assayed using *Saccharomyces carlsbergensis* (Atkins et al., '43). Two grams of liver and intestine and 0.5 gm of kidney were autoclaved with 100 ml of 0.055 N HCl per gram of organ at 15 lb. for 4 hours to release the pyridoxine activity. After cooling and adjusting to pH 4.5, protein and other materials causing turbidity were removed by filtration. Riboflavin was estimated in samples of the organs by the fluorometric method of Loy ('49).

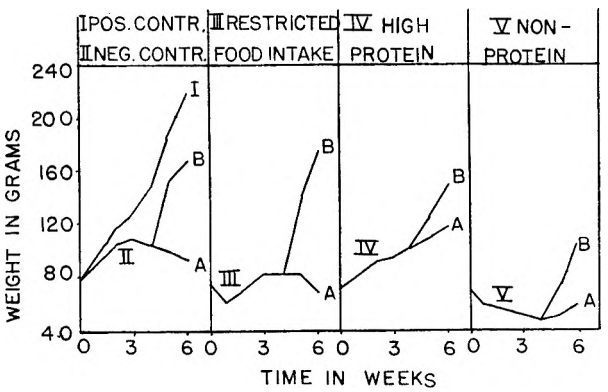

Fig. 1 Effect of the various regimens (± pyridoxine) on weight change in rats.

A = 20% casein ration less vitamin B_6 from 4th to 6th weeks.
B = 20% casein ration plus vitamin B_6 from 4th to 6th weeks.

RESULTS

Growth

During the first 4-week feeding period, it can be seen (fig. 1) that the animals receiving the 20% casein, pyridoxine-deficient ration ad libitum (*negative control,* curve II) reached a plateau and began to lose weight after three weeks. The rats fed the restricted, pyridoxine-deficient ration (*restricted food intake,* curve III) lost weight slightly during the

first week but regained their initial weight and maintained it thereafter over the first 4-week period. The animals receiving the 60% casein, pyridoxine-deficient ration (*high protein,* curve IV) slowly gained weight throughout the first 4-week period. The rats fed the non-protein, pyridoxine-deficient ration (*non-protein,* curve V) lost weight slowly, as would be expected from the lack of protein alone.

In the second phase of the study (4th to 6th weeks), in which all the rats were fed the 20% casein ration ad libitum, supplementation with pyridoxine caused an immediate growth response in all groups (curves IIB to VB). When these responses are calculated as percentage of the control group (curve I) for the same period, the maximum growth response was observed in the initially semi-starved animals (group IIIB). The rats receiving the high protein diet (group IVB) responded least to pyridoxine supplementation. It is interesting to note that the group initially fed the non-protein diet (group VA) responded to the complete ration much less than the initially semi-starved animals. The animals fed the 20% casein ration throughout the experiment (group IIA) gained weight after pyridoxine supplementation to about the same extent as the positive controls.

When the 20% casein-pyridoxine-deficient ration was fed ad libitum from the 4th to 6th weeks (groups IIA to VA), the rats fed the 20% casein ration throughout (group IIA) and those initially semi-starved (group IIIA) lost weight. However the groups initially fed the high-protein diet (group IVA) or the non-protein ration (group VA) gained weight from the 4th to 6th weeks.

Pyridoxine concentration of organs

The effects of the various dietary regimens on the pyridoxine activity and riboflavin concentration of the liver, kidney and intestine are presented in figures 2 to 4. The average pyridoxine values for the organs of the animals in the various groups are indicated by the encircled points. The

range of values for each group is indicated by the horizontal lines below and above each average value.

After 4 weeks of feeding the 20% casein, pyridoxine-deficient ration (group II), the liver, kidney and intestinal pyridoxine activity decreased to 28, 33 and 13%, respectively, of that in the *positive control* group (compare corresponding points in figs. 2 to 4). No further drop in pyridoxine activity was observed in these organs when the same deficient diet was continued for two or more weeks (IIA). Liver, kidney

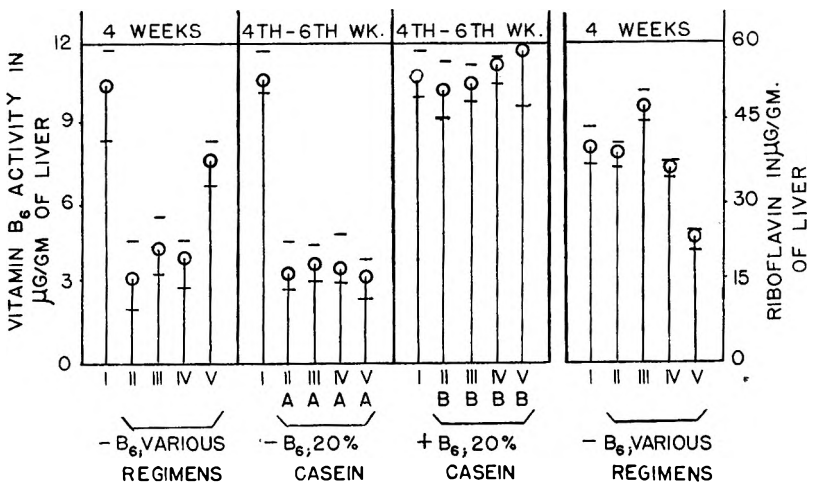

Fig. 2 Results of pyridoxine deficiency and resupplementation on the vitamin B_6 activity and riboflavin concentration of liver tissue of rats.

and intestinal riboflavin was unaffected by the simple pyridoxine deficiency.

The pyridoxine concentrations of the organs of the semi-starved, pyridoxine-deficient rats (group III) followed the same pattern as in group II, both for the first 4 weeks (III) and after shifting to ad libitum feeding (IIIA). The riboflavin content of the liver and of the kidney was unaffected, although intestinal riboflavin was slightly decreased by the pyridoxine deficiency when the animals were semi-starved for 4 weeks.

The rats fed the 60% casein, pyridoxine-deficient diet (group IV) followed the same pattern as those in group II

except that kidney pyridoxine was decreased even further when the ration was changed to 20% casein minus pyridoxine (IVA).

Only a slight decrease in pyridoxine concentration was observed when the rats were fed the non-protein ration (group

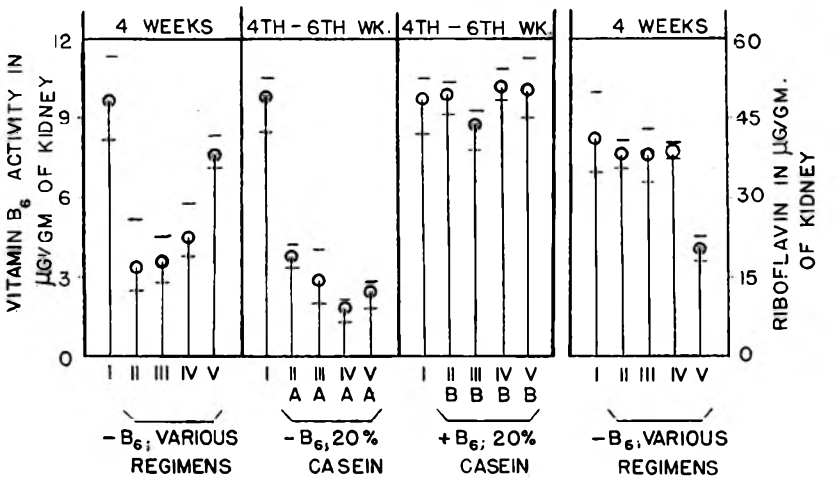

Fig. 3 Results of pyridoxine deficiency and resupplementation on the vitamin B_6 activity and riboflavin concentration of kidney tissue in rats.

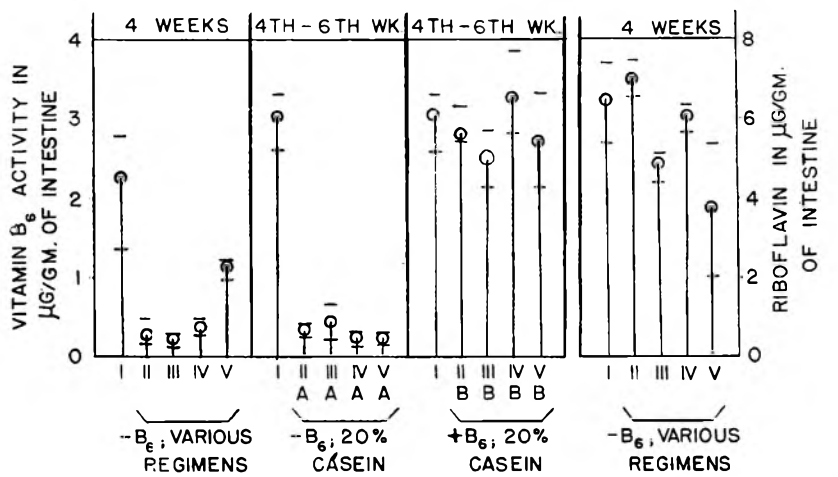

Fig. 4 Results of pyridoxine deficiency and resupplementation on the vitamin B_6 activity and riboflavin concentration of intestine tissue in rats.

V). In this group, after 4 weeks, liver, kidney and intestinal pyridoxine dropped only to 75, 71 and 50%, respectively, of that of the control. When the rats were changed to the 20% casein, pyridoxine-deficient ration (VA), however, the pyridoxine activity for all organs dropped markedly, giving values close to those for the other pyridoxine-deficient groups. It is interesting to note that liver and kidney riboflavin decreased significantly after 4 weeks of feeding the pyridoxine-deficient, non-protein ration.

When the animals of all groups were fed the complete ration ad libitum from the 4th to 6th week (IIB to VB), the pyridoxine activity returned completely to normal for all organs.

DISCUSSION

From the growth response of the rats receiving the 60% casein, pyridoxine-deficient ration, it is evident that growth does not necessarily reflect tissue pyridoxine levels. It is possible that enough of the vitamin was present in the ration, because of the high level of casein, to allow some growth, since the casein contained about 0.5 μg of pyridoxine per gram.[7] This would give about 30 μg of the vitamin per 100 gm of ration, which conceivably is enough to allow the slow rate of growth observed in this group. However, the pyridoxine content of the tissues after 4 weeks had decreased to the same levels as for the groups receiving the 20% casein, pyridoxine-deficient ration or the semi-starvation, pyridoxine-deficient regimen. Thus, high protein apparently does not produce a more pronounced pyridoxine deficiency after 4 weeks than the other regimens, as measured both by growth and tissue concentrations of the vitamin. It would be interesting, however, to study the change in tissue pyridoxine *versus* time. Perhaps a high protein diet would induce a more rapid loss than the other regimens.

When the semi-starved rats were changed to the complete, 20% casein ration and fed ad libitum, the highest rate of

[7] From data kindly supplied by General Biochemical Company, Inc.

weight gain among all of the groups was obtained. This is perhaps accounted for by rehabilitation of body tissues lost during the initial semi-starvation. However, a similar response might have been expected in the rats previously fed the non-protein ration. Since this was not the case, it appears that restricted feeding of an otherwise complete, pyridoxine-deficient diet produces changes which are quite different from those produced by a non-protein diet. The difference in effects of the two diets is further indicated by the fact that the semi-starvation diet produced a greater loss of pyridoxine from the tissues than the non-protein diet. Since the non-protein, pyridoxine-deficient animals held considerable pyridoxine in their tissues after 4 weeks, protein supplementation of the pyridoxine-deficient ration probably accounted for the slow growth observed from the 4th to 6th weeks. Thus the animals could utilize, at least for two weeks, the relatively high levels of pyridoxine in the tissues, when adequate protein was fed. The decrease in tissue pyridoxine concentration during this period indicates that the tissue pyridoxine either was being "used up" when protein was added or that a reapportionment of pyridoxine concentrations among the organs of the body was taking place.

One of the most important conclusions of these studies is that regardless of the previous pyridoxine-deficient dietary regimen (simple pyridoxine deficiency, semi-starvation, high protein regimen, or non-protein regimen), the rat is able to restore tissue pyridoxine concentrations to normal when a complete, 20% casein ration is fed ad libitum for two weeks. These experiments also indicate that, for developing a pyridoxine deficiency over a 4-week period, a 20% "vitamin-free" casein ration is as efficient as a high protein ration.

SUMMARY

1. The effects of various dietary regimens (commonly encountered among human populations) on pyridoxine nutrition have been studied, using the rat. The regimens studied were I, *a normal diet,* II, *a normal diet without pyridoxine,* III,

restricted intake of normal diet without pyridoxine, IV, *high protein diet without pyridoxine,* and V, *non-protein diet without pyridoxine,* all for a 4-week period.

2. Regimen IV, above, was found to produce the same degree of loss of vitamin B_6 activity (approximately 75%) from representative organs of the rat as a 20% level of the same protein (II), after 4 weeks. In this case also, the change in weight was found to correlate poorly with tissue pyridoxine levels.

3. When regimen III was employed, vitamin B_6 in the organs was decreased to the same extent as with diet II.

4. Diet V caused only a 50 to 25% loss of vitamin B_6 in the organs, depending on the one studied.

5. When rats receiving diets II to V for 4 weeks were fed a pyridoxine-deficient, 20% protein ration, no further changes in the vitamin B_6 concentrations of the organs were observed in groups II to IV in two weeks. However, in group V these concentrations were decreased further to the same levels as for the other groups.

6. A return of groups II to V to a complete, 20% protein ration brought all concentrations of vitamin B_6 in the organs to normal within two weeks. During this period, the relative weight gain among the various groups decreased in the following order: III, II, V, and IV.

7. The implications of our findings on the relationships of *extra*-vitamin dietary factors to pyridoxine nutrition have been discussed.

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EXPERIMENTAL OBESITY

I. PRODUCTION OF OBESITY IN RATS BY FEEDING HIGH-FAT DIETS

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THREE FIGURES

(Received for publication July 8, 1955)

Obesity has been produced in the rat by a variety of methods. Although hypothalamic lesions have been used extensively for this purpose (Brobeck et al., '43; Brobeck, '46), these lesions may involve other metabolic disturbances (Mayer, '53). Forced feeding of either normal rats (Wissler et al., '49) or thyroidectomized rats (Scow, '51) results in the deposition of extra fat in the animals.

There are a few indications that obese rats have developed on an ad libitum feeding regimen. Benedict and co-workers ('32) had one rat that reached a weight of 820 gm when fed a purified diet (24% fat) supplemented with brewers' yeast and fresh lettuce. Ingle ('49) reported that rats raised in small cages attained weights as high as 1 kg on a semi-liquid diet with no source of water other than that in the diet.

In order to secure obese laboratory animals as free as possible of other complications, obesity has been produced in normal rats by feeding them a high-fat diet. When this diet is fed on an ad libitum basis to weanling male rats, about 70% of the starting group reach weights close to 1000 gm within 30 to 40 weeks.

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TABLE 1

Composition of purified diets

TYPE OF DIET	HIGH-FAT	HIGH-FAT	HIGH-FAT	LOW-FAT	"BEST" LOW-FAT [1]
DIET NO.	10,001	10,004	10,024	10,010	4419A
	%	%	%	%	%
Casein [2]	25	25	25	30	25
Sucrose	7	6	1	61	65.6
Starch (vitamin B powder)[3]	1	2	2	2	2
Salts [4]	4	4	4	4	4
Crisco [5]	60	60	60	..	..
Cottonseed oil containing:					
Vitamins A and D [6]	2	2	2	2	2
Vitamin E [7]	1	1	1	1	1
Whole liver powder [8]	..	..	5	..	..
Cystine	..	..	..	..	0.3
Aureomycin	..	..	..	..	0.1
Niacin solution 2 mg/ml					1.0 ml
Vitamin B_{12} solution 20 μg/ml					1.0 ml

[1] This diet was developed by Dr. James M. Hundley and Mr. Robert Ing (unpublished data).

[2] Vitamin-free casein from General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Starch containing in each gram: 200 μg thiamine HCl, 300 μg riboflavin, 250 μg pyridoxine HCl, 2 mg calcium pantothenate, 2 mg niacin, 100 mg choline chloride, 10 mg inositol, 1 mg biotin, 100 μg folic acid.

[4] HMW Salts from Nutritional Biochemicals Corp., Cleveland, Ohio.

[5] A hydrogenated fat made by Proctor and Gamble, Cincinnati, Ohio.

[6] Wesson Oil containing 0.5% Natola (secured from Parke-Davis, Inc.). The Natola provides 550 I.U. vitamin A and 110 I.U. vitamin D per 100 gm diet.

[7] Wesson Oil containing 5 mg α-tocopherol acetate per 100 gm diet.

[8] Secured from Wilson and Co., Chicago, Ill.

EXPERIMENTAL

Work with adult rats. In the early stages of this study adult rats were used. Male Sprague-Dawley rats weighing about 300 gm were secured from the Institutes' animal colony. One-third of them were fed a high-fat diet (10,001, table 1), another third were fed a low-fat diet (10,010, table 1), and the remainder were fed a stock diet.[2]

The adult Sprague-Dawley rats on the stock diet reached an average weight of 425 gm after 36 weeks. At that time

[2] Hunt Club dog meal manufactured by Animal Foundation, Inc., Sherburne, N. Y.

the rats on the low-fat diet weighed 417 gm, while those on the high-fat diet weighed 511 gm.

The observation that the rats on the high-fat diet eventually attained higher weights than did those on the other diets suggested the possibility that still higher weights could be secured more rapidly by forced feeding. Although the few rats that survived the forced feeding [3] reached weights of 750 to 800 gm in three to 4 months, the difficulties of the procedure mitigated against its use as a practical means of securing large numbers of obese rats.

More than 75% of the adult Sprague-Dawley rats maintained on the above dietary regimens for 6 months or more developed middle ear or upper respiratory infections.

Work with weanling rats. To reduce the incidence of infection, three- to 4-week-old male rats of the Osborne-Mendel strain were secured from the Institutes' animal colony for the major portion of this work.

Groups of 5 or 10 rats were put on the 63% high-fat diets (table 1), the low-fat diets (table 1), Ingle's ('49) semiliquid diet,[4] and a stock food. The action of penicillin [5] added to these diets was also studied. Since the high caloric density of the high-fat diets might result in a reduced intake of some nutrients, the level of the B vitamins was doubled (diet 10,004). To provide for other nutrients that might be required and to determine their effect on weight gain, the high-fat diets were supplemented with 5% whole liver powder (diet 10,024) and vitamin B_{12}.

A few studies were made to compare the growth rate of NIH black, Sprague-Dawley, and Osborne-Mendel rats on the high-fat diets.

All rats on the above experiments were housed in individual cages with wire-screen bottoms. They were given

[3] Casein in the diet used for stomach tubing was fine enough to pass through a 100 mesh screen. It was prepared by General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Ingle's ('49) diet contained casein, starch, dextrin, sucrose, yeast, salts, vitamins, and water.

[5] Penicillin G sodium was used throughout this work.

food and water ad libitum except for the one group of rats on Ingle's semi-liquid diet which received no water other than that which was in the diet.[6] All diets were kept in a refrigerator except when the food cups were filled.

In order to test Ingle's ('49) suggestion that rats kept under conditions of limited movement become obese, one group of weanling male rats of the Osborne-Mendel strain was placed on Ingle's ('49) diet and another on high fat diet 10,001. Half of the rats on each diet were put in cages that measured $3\frac{1}{2}$ inches wide by $9\frac{1}{2}$ inches long by 7 inches high, the others were maintained in the regular size cages which are twice as wide.

RESULTS

Obese animals developed when weanling male Osborne-Mendel rats were put on any one of a variety of high-fat diets (fig. 1). The composition of the diets could be varied, the primary requisite being that the diets contain adequate amounts of the essential nutrients, due allowance being made for the caloric density of the diets (more than 85% of the calories from fat).

In one of the early experiments the three surviving rats fed one of the high-fat diets (10,004) weighed 790, 1000, and 1116 gm after 41 weeks on the diet (table 2). The heaviest rat on the good, low-fat diet (10,010) weighed 692 gm which was 98 gm less than the lightest one on the high-fat diet. Even when the rats on the low-fat diet were maintained for a total of 71 weeks they never approached the weights of the rats on the high-fat diet. The rats on the stock diet were the smallest.

[6] In our early work the rats received water to drink on an ad libitum basis since Ingle's paper makes no mention of withholding water. He actually emphasized the fact that the obesity was produced "by the ad libitum eating of a diet which is appetizing to the animal." It was only in the later studies with this diet that water was withheld. When drinking water is withheld water must be added to the food cup each day and mixed in with the residual diet. Experience soon showed how much water to add to attain the consistency of the original diet.

Results comparable to the above were secured in other experiments where slight modifications of the high-fat diet were used. One of the ingredients studied at some length was penicillin. The addition of this antibiotic at a level of 40 mg % to a variety of high-fat diets produced no improvement in

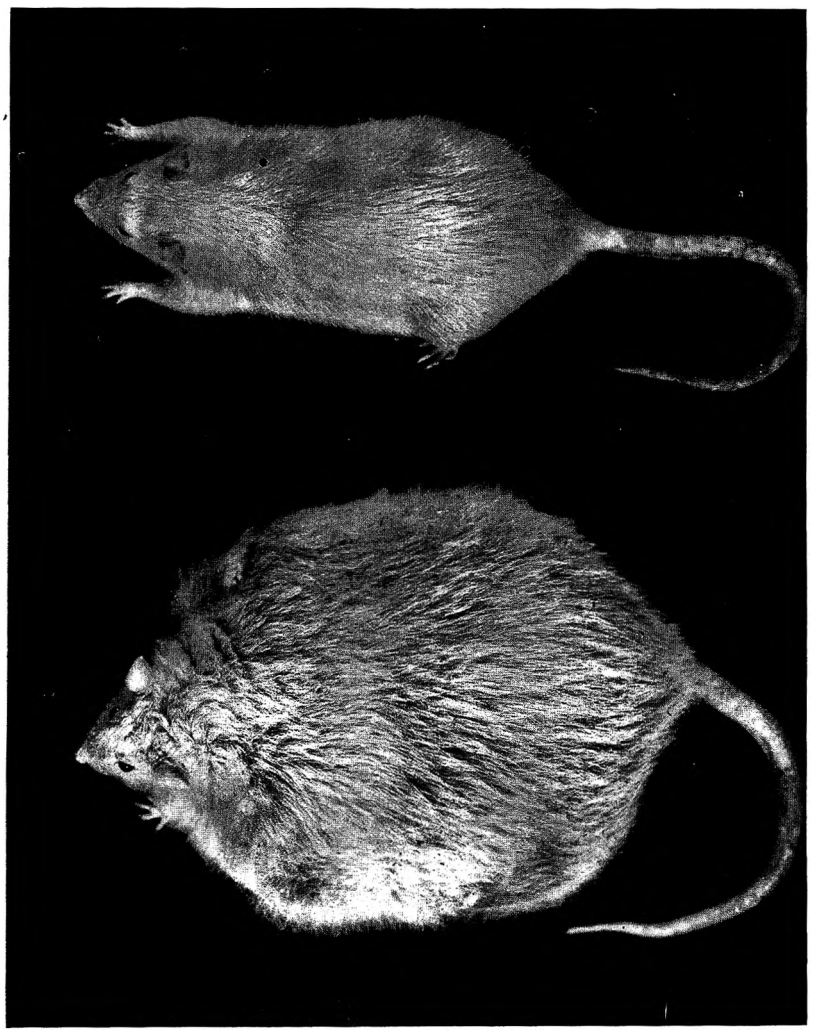

Fig. 1 An obese Osborne-Mendel rat fed high-fat diet 10,024 supplemented with vitamin B_{12} and penicillin, and an age control raised on the stock diet. The obese rat weighs 1445 gm, the control 555 gm.

weight gain. For the combined series the weights of the rats on the high-fat diets averaged 853 gm for the 43 rats which survived out of the 60 started on experiment. The average weights for the penicillin-supplemented series was 890 gm for 50 survivors (60 rats started). The high standard deviations in this series reduced the significance of the difference not only for the entire series but for the individual experiments. Findings similar to these were observed when penicillin was added to the stock and to Ingle's ('49) semi-liquid diets.

Another variation studied was the influence of vitamin B_{12} when added to the high-fat diets. When 2 μg of vitamin B_{12}

TABLE 2

Influence of high-fat diet on weight gains. Weanling Osborne-Mendel rats fed diets ad libitum. All rats were weaned at about three weeks of age. There were 5 rats in each group at the start of the experiment

DIET	WEIGHT AT EXPERIMENTAL WEEK						
	0	5	10	15	25	41	S.E.[2]
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Stock [1]	38	223	327	375	429	504	± 28 (5)
Low fat (10,010)	38	243	373	432	492	579	± 34 (5)
High fat, double vitamins (10,004)	38	239	408	552	726	968	± 109 (3)

[1] Hunt Club dog meal secured from Animal Foundation, Inc., Sherburne, N. Y.
[2] S.E. = Standard error determined by method of Mantel ('51).
( ) Number of survivors at end of experiment.

were added to 100 gm of diet 10,004, the 8 rats remaining on it at the end of the 40th week (10 started on experiment) averaged 724 gm compared to 847 gm for the 8 remaining on the basal diet (10,004). The vitamin B_{12} was tested since work with the chick showed that very high levels of fat in the diet increased the requirement for vitamin B_{12} (Spivey et al., '54). It is recognized that McCollum and Chow ('50) reported that the requirement for vitamin B_{12} was reduced by a high level of fat in the diet.

The substitution of 5 gm of whole liver powder for some of the sucrose (diet 10,024) produced no improvement in

weight gain. The 10 rats on this modification of the diet averaged 807 gm at the 40th week of the experiment compared to 847 gm for the rats on the unsupplemented diet. The whole liver powder has been used in most of our recent diets in spite of the fact that it did not increase the weight gain of the rats. It is a good source of some of the nutrients which might be unknown and it also provides about 3 gm of extra protein of a high biological value per 100 gm of diet.

The 10 rats kept on Ingle's ('49) diet (plus an additional source of water) in regular size cages weighed 666 gm after 44 weeks on the diet, while rats on the high-fat diet (10,001) in the same size cages weighed 661 gm. The 9 surviving rats on Ingle's diet kept in half size cages weighed 626 gm, while rats on the high fat diet (10,001) kept in the smaller cages weighed 665 gm. These results show that there was no difference in the final weights attained whether the rats were confined in small or regular size cages. The width of the obese rats is such (fig. 1) that the smaller cages might produce abrasions of the skin with subsequent infection. Furthermore, none of the obese rats showed enough voluntary activity to suggest any appreciable caloric saving as a result of restricting their already limited movements. The small size cages are difficult to clean and to service. For these reasons the use of these cages was abandoned.

In the experiments where the only source of water for the rats on Ingle's ('49) diet was that in the diet, 7 rats surviving at the 41st week (out of 10 started) averaged 787 gm. The 8 survivors on the high-fat diet (10,004) averaged 924 gm. The differences in final weights between the rats on Ingle's semi-liquid diet with no additional water and the rats on the high-fat diet or variations thereof have ranged from 0 to 140 gm. The evidence indicates that with the Osborne-Mendel strain of rat the high-fat diet produces as obese animals as the technique proposed for this purpose by Ingle ('49).

The weanling NIH black rats fed a high-fat diet (10,004) attained an average weight of 356 gm after 25 weeks on the diet. In another experiment the black rats on the high-fat

diet weighed 556 gm after 50 weeks, while those on the stock diet weighed 377 gm.

Although the Osborne-Mendel rats were the same weight as the Sprague-Dawley at weaning, the former weighed 666 gm after being on high-fat diet 10,004 for 25 weeks, while the Sprague-Dawley rats at that time weighed 464 gm.

Since there did not appear to be differences in the degree or incidence of obesity produced by any of the high-fat diets used in these studies, all rats fed any of the high-fat diets were pooled for the following: more than 60 rats (about 35% of the starting group) attained weights above 1000 gm. An equal number of rats reached weights between 800 and 1000 gm. On this basis approximately 70% of the rats fed the high-fat diets became obese. The heaviest one in this work to date weighed 1655 gm compared with 555 gm for its control on the stock diet.

The growth curves for the individual Osborne-Mendel rats on the high-fat diet (fig. 2) showed only a small inter-individual variation in rate of gain through the 10th week of the experiment. After that there were marked individual differences both in the rate of gain and in the final weights attained. The curves in figure 2 represent the growth rates of the three heaviest and the three lightest animals from a randomly selected group of 10. The heaviest rat in this group weighed 1290 gm. In this experiment 6 of the 10 rats exceeded 1000 gm, while the other 4 exceeded 800 gm. A large proportion of the rats attained their maximum weights between the 40th and 50th weeks on experiment. After that some rats showed a plateau in body weight. Eventually, however, they started to lose weight. The rate of weight loss in the beginning was sometimes slow but finally the rats lost 30 to 50 gm per week. When this happened they usally died within a short time. Some of the rats started to lose weight after reaching the peak in their growth curves without an intervening plateau.

The weight curves (fig. 3) for 6 of the rats on the "best" low-fat diet show that there was a break at about the 15th

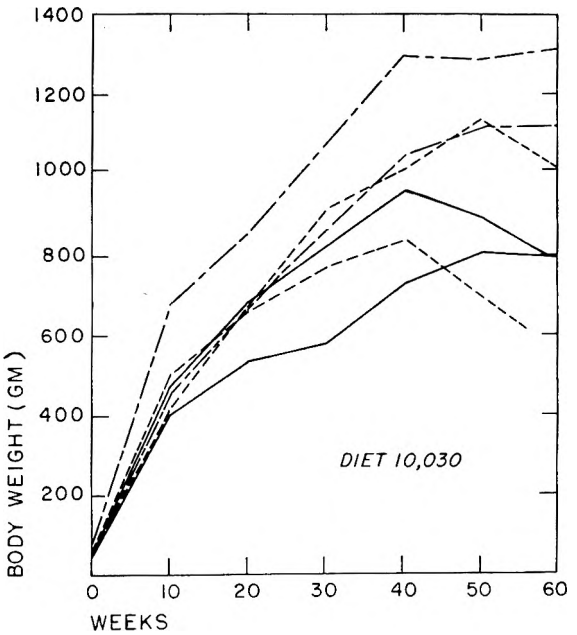

Fig. 2 Growth curves for individual Osborne-Mendel rats raised on a high-fat diet (10,030 = 10,024 + penicillin + vitamin B_{12}). The curves for the three heaviest and the three lightest rats in one experimental group are plotted.

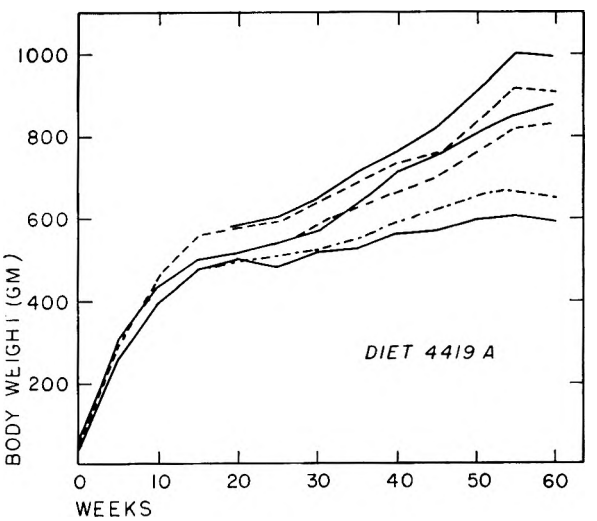

Fig. 3 Growth curves for individual Osborne-Mendel rats maintained on the "best" low-fat diet 4419 A. The curves for the 4 rats weighing the most at the end of the experiment and the two weighing the least are plotted.

week of the experiment. At that time some of the rats started gaining slowly and continued to gain for almost a year. Other rats, however, showed a spurt in body weight gain which began between the 20th and 30th weeks of the experiment. The spurt was observed at a similar period in other groups raised on different low-fat diets. The rats which showed the spurt in weight gain continued to increase until some of them approached 1000 gm. On the basis of a body weight considerably heavier than that of normal adults, these animals were obese. The maximum weight attained in these animals occurred only when the rats were about a year old.

The rats on the stock diet gained weight at a slower rate than those on either the low- or high-fat diets and did not exceed a weight of 660 gm even after one year. Their growth curves showed a plateau similar to those for the lightest rats in figure 2.

DISCUSSION

Obesity has been produced in about 70% of normal weanling rats fed a high-fat diet on an ad libitum basis. This is one method for producing obesity by purely dietary means. The obese rats are about 1 cm longer than the controls on the stock diet (as determined by nose to anus length). They are, however, 1.5 to 2.5 times as heavy. These animals should prove useful in studying various problems associated with obesity or a weight reduction subsequent thereto.[7]

We have no explanation as to why rats fed any of the above high-fat diets were unable to adjust their caloric intake to their requirements. Of the three strains studied the Osborne-Mendel rats attained the highest weights. One might suggest that the Osborne-Mendel rats have an inherent tendency to become obese. If that were true, they might be compared to the C3H mice which were shown by Fenton and co-workers ('51a, '51b, '53) to become obese when fed a 50% fat diet. Mice of other strains did not respond in the same manner. However,

[7] Weight reduction has been accomplished using some of the obese rats weighing approximately 1000 gm. Work on this phase of the study will be reported subsequently.

it is unlikely that the obesity which developed in the Osborne-Mendel rats fed high-fat diets was due to a genetic variation. There has been no report of an inherent abnormality in the NIH black, Sprague-Dawley, and Osborne-Mendel strains which results in obesity. Furthermore, all three strains, when fed the high-fat diets, reached weights which were 1.5 to 2.5 times those seen in rats fed the stock diet. A small percentage of the rats maintained on one of the low-fat diets for periods of a year or more reached weights close to 1000 gm. The rate at which the latter animals gained was slower and the final weights attained were lower than those for rats on the high-fat diets.

At first glance it appears surprising that so many rats became fat when fed ad libitum. It is claimed that animals do not develop obesity under normal conditions (Mayer, '53). Actually, many domesticated animals, especially older ones, are likely to get considerably heavier than the average adult weight. This is the case where the need for physical activity is markedly reduced and abundant amounts of highly nutritious diet are available. To a certain extent the above is comparable to the rats on the "best" low-fat diet. Some of those rats eventually attained weights close to 1000 gm (fig. 3). These animals on the low-fat diet gradually increased in weight after an initial levelling off in early adulthood.

The present study indicates that when rats of a rapidly growing strain are fed a "luxus" diet containing a high percentage of fat, a large number of the animals attain weights which are about twice those normally seen in adults. The high-fat diets appear to increase both the rate at which obesity is developed and the maximum weight attained. The rats grow to extreme weights without any sign of ketosis even though the diet provides more than 85% of the calories from fat.

A number of reports have appeared on the effect of feeding rats high-fat diets. In none of these cases has there been any indication that the animals become obese. Some investigators reported that the rats on their high-fat diets grew

faster than did the animals on the diet containing a lower level of fat (Reed et al., '30; Hoagland and Snider, '40; Haldi et al., '42; Lundback and Stevenson, '47; Hoagland et al., '52). Others noticed no change in the growth rate of the rats on their high-fat diets (MacKay and Sherrill, '41; Deuel et al., '47; Vitale et al., '51; Strominger et al., '53). In many of these experiments the protein levels in the high-fat diets were those that would be adequate for rats fed low-fat diets. Because of the caloric density of high-fat diets, they contain less protein per 100 calories. Since the rats on high-fat diets do not consume as many grams of food as those on low-fat diets, their protein intake in the preceding reports might have been inadequate for normal growth. This and the short experimental periods may explain the absence of obesity in these studies.

It has been suggested that the rats on our high-fat diets became obese because they ate more than their caloric requirement in order to overcome some dietary deficiency. The work of Johnson et al. ('36) has been mentioned in support of such a theory. Actually Johnson et al. ('36) found that when weanling rats were put on a low-protein diet, they stored slightly more fat (5.2 gm) than did those on a normal protein intake (2.8 gm). The small absolute differences in the fat content of these rats are probably of no consequence in explaining the marked differences in body weight of the rats on our high-fat diets compared to the stock controls.

The preceding hypothesis implies that our obese rats have consumed excessive amounts of the high-fat diets to overcome a dietary deficiency. If this were so, then one would expect either a reduction in weight or a lower maximum weight upon correction of the deficiency. Every attempt to improve the high-fat diet by increasing the protein level (by that in the 5% whole liver powder), by adding extra vitamins (doubling the water soluble vitamins and adding vitamin B_{12}), and by adding the possible growth factors in whole liver powder has not reduced either the rate of gain or the maximum weight attained by the rats.

SUMMARY

1. Obesity has been produced in normal male rats by the ad libitum feeding of a diet containing 63% of fat and adequate amounts of vitamins, minerals and protein.

2. When weanling rats were fed this diet, they gained weight at a higher rate than rats on our "best" low-fat or stock diets. Three strains of rats have shown the same response. The maximum weight attained on the high-fat diet was 1655 gm.

3. Approximately 70% of the weanling rats of the Osborne-Mendel strain randomly secured from the stock colony have attained weights over or close to 1000 gm when fed the high-fat diet. We believe that the obesity does not result from any genetic or hormonal disturbance.

ACKNOWLEDGMENTS

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THE EFFECTS OF CALCIUM AND PHOSPHATE IN FOODS ON RADIOSTRONTIUM ACCUMULATION [1]

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ONE FIGURE

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One of the potential hazards to health, which is demanding attention since the widespread dissemination and use of radioactive materials, is that resulting from the accidental ingestion of the strontium isotopes, Sr^{89} and Sr^{90}. These isotopes are readily absorbed from contaminated food or water and are deposited in the skeleton, where they are retained for very long periods (Hamilton, '47). Their beta particle emissions can seriously impair the blood-forming functions of bone marrow and can induce bone tumors, if present in sufficient quantity (Brues et al., '47). Several materials have been shown to reduce the deposition of milligram doses of nonradioactive strontium when mixed with it and administered orally to rats. Phosphate was one such agent (MacDonald et al., '52). Oral doses of natural, stable strontium have been suggested as a therapeutic measure for radiostrontium poisoning (Hamilton et al., '47; Gross et al., '53).

Because of their similarity in metabolic behavior, it seemed reasonable that calcium also might affect the absorption and retention of radiostrontium. That is, calcium might act as a "biological carrier" for Sr^{90}. Indeed, an inhibitory effect

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on radiostrontium retention afforded by the maintenance of a high level of dietary calcium, *prior* to administration of the radioisotope, has been reported (Copp et al., '47; Kidman et al., '50). The purpose of the work reported here was to determine what effect, if any, the calcium and phosphate levels of various foods would have on the skeletal accumulation of Sr^{90} put into the food.

EXPERIMENTAL PROCEDURES

Male albino rats were segregated into groups of 30. Food was withheld for 24 hours. Sub-groups of 10 animals were then administered Sr^{90}–Y^{90} along with 2.0 ml of the test food slurry by gavage. A control sub-group received the same amount of radiostrontium mixed with distilled water or strained beef. The suspensions were prepared by stirring each powdered agent in distilled water. The radiostrontium was administered as 0.20 ml of a water solution of carrier-free Sr^{90} chloride in radioactive equilibrium with its daughter, Y^{90}. This represented an activity of about 3680 disintegrations per second, as determined by dilution of this volume and the counting of evaporated aliquots removed therefrom. After 48 hours, the animals were sacrificed, the femurs removed, ashed, and dissolved in acid. Thereafter, an aliquot was evaporated and counted with a thin-window Geiger-Muller tube and conventional scaling circuit.

Calcium determinations were performed by precipitation of the oxalate salt followed by titration with standard permanganate. The phosphate analyses were made by spectrophotometric determination of the molybdivanadophosphate complex according to the technique of Gee and Deitz ('53).

RESULTS AND DISCUSSION

Table 1 summarizes the results of the radiostrontium counting data. The last column shows the effect of the material which accompanied the radioisotope on the fraction of the Sr^{90} dose which was found in both femurs.

TABLE 1

The effects of various agents on accumulation of Sr^{90} in rat bone

SERIES NO.	TREATMENT	WEIGHT RANGE	PER CENT OF Sr^{90} DOSE IN FEMURS [1]	P VALUE [2]	EFFECT [3]
		gm			
1	Regular wheat cereal [4]	190–215	1.96 ± 0.28	0.4	n.s.
2	Enriched wheat cereal [4]		1.61 ± 0.14	..	n.s.
	Controls: water [5]		1.62 ± 0.27	..	...
3	Regular oats [6]	195–225	1.50 ± 0.20	..	n.s.
4	Enriched oats [7]		1.00 ± 0.16	0.05	— 42
	Controls: water		1.61 ± 0.24	...	...
5	Green beans, strained	185–215	1.99 ± 0.17	..	n.s.
6	Spinach, strained		1.61 ± 0.26	0.2	— 21 (?)
	Controls: water		2.04 ± 0.17	..	...
7	Orange juice, canned	185–220	1.96 ± 0.24	..	n.s.
8	Beef, strained		1.71 ± 0.29	0.3	n.s.
9	EDTA [8]		2.55 ± 0.32	0.2	+ 23 (?)
	Controls: water		2.07 ± 0.16	..	...
10	Rat diet [9]	200–218	2.27 ± 0.14	0.01	— 30
11	Milk powder [10]		3.20 ± 0.26	...	n.s.
	Controls: water		3.25 ± 0.20	...	...
12	Milk powder [10]	190–220	2.35 ± 0.15	0.01	+ 41
13	Milk powder [10] + EDTA		2.12 ± 0.31	0.2	+ 27 (?)
	Controls: water		1.66 ± 0.11	..	...
14	Milk powder [10]	195–215	1.93 ± 0.20	..	n.s.
15	EDTA		2.72 ± 0.40	..	n.s.
	Controls: water		2.16 ± 0.40	..	...
16	Milk powder [11]	215–240	1.51 ± 0.21	..	n.s.
	Controls: water		1.27 ± 0.31	..	...
17	Whole milk, homogenized	217–230	0.88 ± 0.06	0.01	— 52
	Controls: water		1.82 ± 0.14	..	...
18	"Fat-free" milk	230–245	1.80 ± 0.18	0.2	— 21 (?)
	Controls: water		2.28 ± 0.31	..	...
19	Na_3PO_4	240–260	0.97 ± 0.14	0.01	— 60
20	$CaCl_2$		1.91 ± 0.22	0.35	n.s. (?)
	Controls: water		2.42 ± 0.45	..	...
21	Na_2HPO_4	235–250	1.44 ± 0.32	0.2	— 39 (?)
22	Na_2SO_4		1.56 ± 0.20	0.2	— 34 (?)
	Controls: water		2.38 ± 0.59	..	...
23	Beef, strained + $CaCl_2$ (20 mg Ca)	190–210	2.49 ± 0.14	0.1	— 26
24	Beef, strained + $Ca_3(PO_4)_2$ (20 mg Ca)		2.05 ± 0.12	0.02	— 39
	Controls: beef, strained		3.37 ± 0.34	..	...
25	Beef, strained + $CaCl_2$ (100 mg Ca)	190–225	2.25 ± 0.16	0.01	— 32
26	Beef, strained + $Ca_3(PO_4)_2$ (100 mg Ca)		1.31 ± 0.12	0.01	— 60
	Controls: beef, strained		3.29 ± 0.14	..	...
27	Beef, strained + $CaCl_2$ (200 mg Ca)	185–220	1.74 ± 0.10	0.01	— 49
28	Beef, strained + $Ca_3(PO_4)_2$ (200 mg Ca)		0.98 ± 0.09	0.01	— 72
	Controls: beef, strained		3.46 ± 0.32	..	...

[1] Mean percentage of the Sr^{90}-Y^{90} dose which was found in both femurs ± the standard error.
[2] The P value for the statistical significance ("t" test) of the difference between the control mean and the treated mean values.
[3] Effect of the test treatment expressed in % of control value: (Test mean — control mean) (100) ÷ (Control mean).
[4] Cream of wheat.
[5] When water was used as the control agent, 2.0 ml were given.
[6] Quaker Oats
[7] Pablum.
[8] A solution of the disodium salt of ethylene diamine tetra acetic acid, adjusted to pH 6.3.
[9] Rockland.
[10] Starlac.
[11] In this series, 1.5 gm of milk powder (Starlac) were given = twice the dose used in series 11, 12, 13 and 14.

Table 2 sets forth a grouping of the results according to the molar doses of calcium and phosphate or other anion. The agent, EDTA,[2] was omitted since it contained no calcium or phosphate. If its slight enchancing effect is real (series 9, 13 and 15), this might be explained as the result of increased absorption from the gut via the Sr^{90} chelate, followed by displacement of the Sr^{90} from the complex by serum Ca and deposition of this liberated radiostrontium in the bone.

There was a definite reduction in bone accumulation of ingested Sr^{90} when mixed with materials in which the calcium level was elevated. The reduction was even more pronounced when the phosphate was also elevated. The various milk products tested were notable exceptions to this generalization.

The influences of the phytic acid content of the wheat and oat products and the oxalic acid content of the spinach remain undetermined at the present time. The same uncertainty applies to the possible action of vitamin D which may have been present in the agents. Nevertheless, the reduction of Sr^{90} bone burden with increase of ingested calcium and phosphate is clear.

If one considers the series 23, 25 and 27, in which different amounts of $CaCl_2$ were mixed with a dose of beef and Sr^{90}, the influence of Ca^{++} alone is more readily discernable. Figure 1 presents these data on a semi-logarithmic plot. The fraction of the Sr^{90} dose which was found in the bone became smaller as amount of accompanying calcium was raised, but progressively larger and larger doses of calcium were required to maintain this trend. There are three lines of speculation which may be directed to this experimental finding:

1. If the quantity of calcium had exerted *no* influence on the accumulation of Sr^{90}, then there should have been no observable change in bone burden of Sr^{90}, since the doses of radioisotope were the same.

[2] A solution of the disodium salt of ethylene diamine tetra acetic acid, adjusted to pH 6.3.

TABLE 2

Doses of materials accompanying radiostrontium administration

AGENT	SERIES NO.	AMOUNT GIVEN [1]	Ca^{++} DOSE [2]	PO_4^{--} DOSE [2]	EFFECT	DOSE CATEGORY [3]
Beef + $Ca_3(PO_4)_2$	28	2 ml beef + 1 ml Ca solution	500	333	— 72	
Beef + $Ca_3(PO_4)_2$	26	2 ml beef + 1 ml Ca solution	250	173	— 60	
Beef + $Ca_3(PO_4)_2$	24	2 ml beef + 1 ml Ca solution	50	40	— 39	High Ca High PO_4
Milk powder [4]	16	1.50 gm	55	38	n.s.	
Milk powder [4]	14	0.75 gm	27	19	n.s.	
Milk powder [4]	12	0.75 gm	27	19	+ 41 (?)	
Milk powder [4]	11	0.75 gm	27	19	n.s.	
Rat diet [5]	10	0.75 gm	21	18	— 30	
Beef + $CaCl_2$	27	2 ml beef + 1 ml Ca solution	500	7	— 49	
Beef + $CaCl_2$	25	2 ml beef + 1 ml Ca solution	250	7	— 32	High Ca
Beef + $CaCl_2$	23	2 ml beef + 1 ml Ca solution	50	7	— 26	Low PO_4
$CaCl_2$	20	0.377 gm	340	...	n.s. (?)	
Enriched oats [6]	4	0.50 gm	4	11	— 42	
Na_3PO_4	19	0.56 gm	0	340	— 60	Low Ca
Na_2HPO_4	21	0.48 gm	0	340 (as HPO_4^{--})	— 39	High anion
Na_2SO_4	22	0.48 gm	0	340 (as SO_4^{--})	— 34	
Enriched wheat cereal [7]	2	0.75 gm	8	5	n.s.	
Regular wheat cereal [7]	1	0.75 gm	0.3	3	n.s.	
Regular oats [8]	3	0.50 gm	0.3	7	n.s.	
Green beans, strained	5	2.0 ml	2	3	n.s.	Low Ca Low PO_4
Spinach, strained	6	2.0 ml	8	6	— 21 (?)	
Beef, strained	8	2.0 ml	0.4	6	n.s.	
Orange juice, canned	7	2.0 ml	0	0.8	n.s.	
Milk, whole, homogenized	17	2.0 ml	6	6	— 52	
Milk, fat free	18	2.0 ml	6	6	— 21 (?)	

[1] When the amount given is expressed in grams, this weight was suspended in 2 ml of distilled water.

[2] Expressed in moles $\times 10^{-5}$.

[3] A high dose is arbitrarily defined here as more than 10×10^{-5} moles of either Ca or PO_4; a low dose is less than this amount.

[4] Starlac.

[5] Rockland.

[6] Pablum.

[7] Cream of wheat.

[8] Quaker Oats.

2. If the calcium had acted merely as an *inert diluent,* mechanically decreasing the opportunities for Sr^{90} atoms to be absorbed during passage of the administered material through the intestinal tract, then the changes in bone burden should have been much more pronounced. For example, the

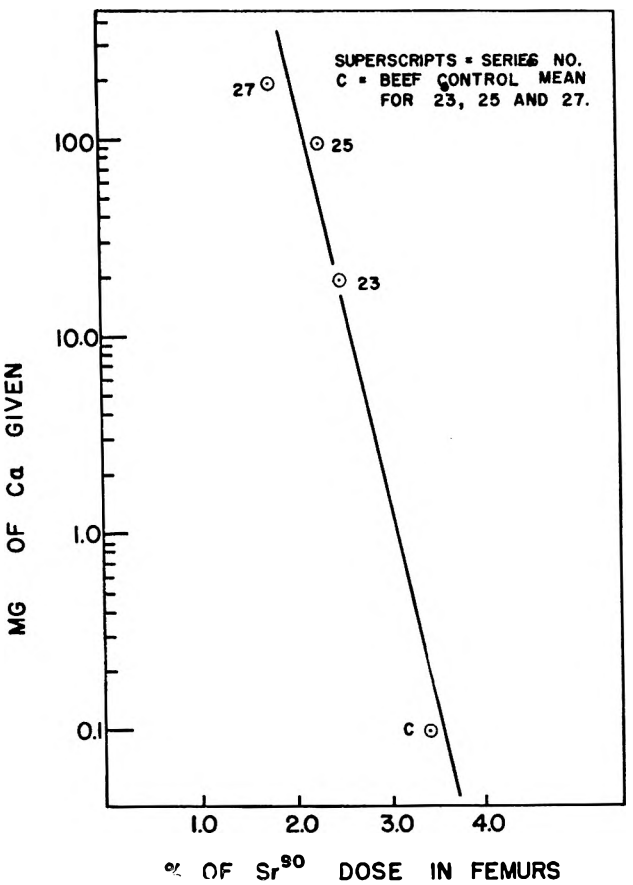

Fig. 1 Effect of $CaCl_2$ on uptake of Sr^{90} mixed with beef-water slurry.

Sr^{90} to Ca ratio in series 27 was only 0.05% of the Sr^{90} to Ca ratio of the control material, yet the retention of Sr^{90} was 51% of the control retention.

3. If the Sr^{90} had been treated qualitatively in the same fashion as calcium during digestion, then the effect of calcium

dose on Sr^{90} retention in figure 1 is also indicative of the utilization of the ingested calcium. The assumption here is that the metabolic fates of strontium and calcium are almost identical, in spite of possible quantitative differences in such factors as ease of transport across biological membranes, for example. In this light, the relatively few Sr^{90} atoms, which were mixed with the very large number of Ca atoms, behaved as tracers for calcium movements. This interpretation is compatible with the commonplace observation that the accumulation of calcium in the skeleton is partly governed by the amount provided. Thus, within the range of dosage used in the present experiments, the actual *amount* of calcium retained in bone increased as the dose was increased, but the percentage of the administered calcium which was utilized, decreased. Whether or not the percentage utilized is truly a logarithmic function of the amount of calcium presented to the digestive organs cannot be determined from the inadequate data in this experiment. It should be noted here that experiments now in progress, together with a few allusions to the matter in the literature, indicate that strontium is not a perfect biological tracer for calcium. There are small quantitative differences in their metabolic behavior. None the less, the inhibitory effect of calcium on radiostrontium retention, under the conditions of these experiments, may be ascribed to the "carrier" action of the former ion. That is, the greater the amount of calcium accompanying a fixed quantity of Sr^{90}, the greater the dilution of the radioactive ions with respect to total alkaline earth ions and therefore, the smaller the fraction of the Sr^{90} dose which is absorbed and retained.

Lacking direct evidence, it is not unreasonable to attribute the inhibitory effect of phosphate ion to a precipitating action on Sr^{90} in the gut. The reduced bone burden is thus a reflection of the decrease in absorption of Sr^{90} ions from the intestine. This mechanism is strongly supported by the similar effects

of HPO_4 and SO_4 which also form difficultly soluble precipitates with strontium ions *in vitro*.

Further experiments to elucidate the anomalous behavior of the various milk products and EDTA are to be performed. Meanwhile, the authors suggest that the hazards of ingestion of foods which might have become contaminated with radiostrontium are significantly lessened by the calcium and phosphate in the foods naturally or by virtue of intentional enrichment.

SUMMARY

Each of a series of common food materials, accompanied by a fixed dose of Sr^{90}–Y^{90}, was administered by gavage to rats. The femurs were removed 48 hours later and analysed for radiostrontium activity. The calcium and phosphate content of each of the materials was also determined.

There was a clearcut reduction of Sr^{90} burden with increase of ingested calcium. This reduction was even more pronounced when the phosphate as well as the calcium dose was elevated.

Some speculations concerning the inter-relationships of orally administered calcium and Sr^{90} were presented. Assuming that Sr^{90} acted as a "tracer" for calcium, the results implied that the *percentage* of an oral dose of calcium which deposits in the skeleton, decreases as the size of the dose increases.

It was suggested that the potential hazards from ingestion of foods contaminated with radiostrontium are somewhat diminished in proportion to the calcium and phosphate concentrations existing in the foods naturally or by enrichment.

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UTILIZATION OF SWEET POTATO STARCH BY RATS AND ITS EFFECT ON THE DIGESTION OF DIETARY PROTEIN

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It is known that the degree of protein utilization is influenced by the nature of carbohydrate in the diet. Monson et al. ('50) observed that chicks on a dextrin diet grew markedly faster than those on a sucrose diet and that the dextrin diet passed through the digestive tract of chicks more slowly than a sucrose or lactose diet. Monson et al. ('54) also have reported that chicks receiving dextrin grew more rapidly than those receiving sucrose when the level of casein was 18% or less. However, when the casein content of the sucrose diets was increased above 18%, growth approached that of chicks receiving 18% casein with dextrin. These authors suggested that dextrin exerts this effect largely by permitting better utilization of dietary protein. Several Wisconsin workers have reported the superiority of dextrin to sucrose as a source of carbohydrate in the diet of rats (Krehl et al., '46; Henderson et al., '47; Hankes et al., '48; Lyman and Elvehjem, '51), and the increase of xanthine oxidase activity in the liver of rats fed dextrin in the place of sucrose in the diets (Kring and Williams, '51; Harper et al., '53). Harper and Katayama ('53) reported that rats fed 9% of casein grew faster when cornstarch replaced sucrose as the dietary carbohydrate. Harper et al. ('53) reported further studies along this line and they suggested that the utilization

of dietary protein is improved when the sucrose in low-protein diets is replaced by dextrin. Marshall and Womack ('54) have shown that rats fed 0.1% of essential amino acids with sucrose had more negative nitrogen balances than rats fed dextrin. Recently, Dreisbach and Nasset ('54) have compared the absorption rates of glucose arising from corn starch, banana meal and glucose and have reported that absorption of glucose arising from cornstarch is the slowest.

On the other hand, it has been shown that certain starches, mainly those derived from tubers, are poorly utilized by animals. Booher et al. ('51) have reported that cereal starches are assimilated nearly perfectly and those from arrowroot, white potato and sago palm showed low degrees of digestibility. They suggested that digestion-resistant properties of the latter reside in the outermost layer of granules. Sweet potato starch occupied an intermediate position in their results. Jelinek et al. ('52) have reported the inadequacy of unmodified potato starch as a source of dietary carbohydrate for rats and the improvement of its utilization by several treatments. Harper et al. ('52) have shown that raw and autoclaved potato starch in the diet induced a relatively large excretion of endogenous nitrogen and amino acids.

It is the purpose of this paper to report a comparison of the digestibility of sweet potato starch with that of corn starch and potato starch, and to study the effect of these starches upon the digestion of dietary protein. Also some further data on the effect of steaming and purification of sweet potato starch upon digestion are presented.

EXPERIMENTAL

Design of experiment. The experiment was composed of three series. Series 1 consisted of a comparison of the digestibility of three kinds of starch, i.e. corn, sweet potato and potato starch. Eight male rats of Wistar strain, weighing 300 to 400 gm, were housed in individual cages with raised screen floors. The feeding plan is shown in the first 5 columns of table 2. Each diet was fed, with the free supply of water,

for 10 days and the feces were collected on a screen set under the cage for the latter half of each period. Fifteen grams of diet were given to each rat daily and all of the food was consumed. Raw diet was given mixed with water, steamed diet was mashed and steamed for 15 minutes. Test 7 was done in May 1954 and was designed to check the seasonal change in digestibility, the rats being fed the same diet as that in test 1 which was finished in January 1954.

The effect of purification of sweet potato starch was studied in series 2, using 6 rats of 230 to 310 gm body weight. This series consisted of a comparison of the digestibility of raw cornstarch and three kinds of sweet potato starch, i.e. raw, purified and autoclaved, in the same manner as in series 1, except that the daily food intake was 18 gm. It is supposed that steaming of the diet decomposed some of vitamins and protein. To avoid this, sweet potato starch was autoclaved in the dry state at 110°C. for 24 hours, powdered after cooling and mixed with the other nutrients. The diets in series 2 were given mixed with water.

In series 3, metabolic fecal nitrogen was determined by the method advocated by Mitchell ('24). The same rats as those of series 2 were fed two kinds of low protein diet, one of which contained raw cornstarch as the carbohydrate source, and the other, raw sweet potato starch. Five per cent of whole egg powder was given as the protein source in both diets. Eighteen grams of diet were given daily mixed with water.

Starch. Starches used were the first grade commercial starches, the chemical composition of which is shown in table 1. In series 2, sweet potato starch was suspended and settled 4 times in distilled water. The upper and lower layers were discarded and the starch was washed twice with alcohol and once with ether, suspended and settled 12 times with 0.2% sodium hydroxide and washed with distilled water till supernatant became neutral.

Diet. The percentage composition of the diets fed in series 1 was as follows: starch 65, butter fat 10, casein 10, fish meal 5, brewers' yeast 4, cellulose (flour of filter paper)

3, McCollum's salt mixture no. 185 3. In series 2 and 3, whole egg powder was used as protein source instead of the mixture of casein, fish meal and yeast in series 1. The percentage composition of the diets fed in series 2 was as follows: starch 68, whole egg powder 23, butter fat 2, cellulose 3, salt mixture 3, and vitamin mixture 1. The low protein diet used in series 3 contained 77% starch, 5% whole egg powder, 10% butter fat, 3% cellulose, 4% salt mixture, and 1% vitamin mixture. One gram of vitamin mixture contained 2,500 I.U. of vitamin A, 200 I.U. of vitamin D, 1.0 mg of thiamine, 1.5 mg of riboflavin, 10 mg of niacin, 1.0 mg of pyridoxine, 0.5 mg of folic acid, 0.5 mg calcium pantothenate,

TABLE 1

Chemical composition of starch

STARCH	MOISTURE	NITROGEN FREE EXTRACT	TOTAL OTHERS DETERMINED
	%	%	%
CS [1]	14.15	85.61	0.24
SPS	16.61	83.01	0.38
SPS (purified)	16.88	82.99	0.13
PS	18.46	81.24	0.30

[1] CS = cornstarch; SPS = sweet potato starch; PS = potato starch.

1.0 μg of vitamin B_{12}, 1.0 mg of vitamin E, 0.2 mg of vitamin K, and 37.5 mg of ascorbic acid. Ingredients other than starch showed no starch-reaction with iodine.

Analytical methods. The feces of each rat were collected for 5 days and were air-dried and powdered. Their starch and nitrogen contents were analyzed by the following method: the sample was hydrolyzed with 0.6 N hydrochloric acid, neutralized with 0.5 N sodium hydroxide and centrifuged. The reducibility of supernatant solution was determined iodometrically with Somogyi's phosphate reagent ('45). In series 1, starch content was determined at the same time by the method of Pucher et al. ('48). Starch was extracted with perchloric acid, precipitated as starch-iodine complex, liberated by alcoholic sodium hydroxide and de-

TABLE 2

The digestibility of three kinds of starch

SERIES	TEST NO.	DIET			WEIGHT [1] OF FECES	NITROGEN			STARCH	
		Source of carbohydrate	Content			Daily intake	Daily output	Apparent [2] digestibility coefficient	Daily intake	Apparent [2] digestibility coefficient
			Protein	Starch						
			%	%	*gm*	*mg*	*mg*	%	*gm*	%
1	1	Raw CS [3]	15.8	50.9	1.25	378	43.2	88.6 ± 0.69	7.6	99.3 ± 0.04
	2	Raw SPS	15.6	50.1	1.76	374	75.7	79.8 ± 1.37	7.5	96.8 ± 1.21
	6	Raw PS	14.7	48.9	5.65	354	93.0	73.7 ± 1.62	7.3	57.2 ± 6.11
	3	Steamed CS	15.8	50.9	1.37	378	52.2	86.3 ± 1.35	7.6	98.9 ± 0.16
	4	Steamed SPS	15.6	50.1	1.28	374	48.0	87.2 ± 0.77	7.5	99.2 ± 0.09
	5	Steamed PS	14.7	48.9	1.34	354	48.8	86.2 ± 1.12	7.3	99.0 ± 0.06
	7	Raw CS	15.8	50.9	1.27	378	43.1	88.6 ± 0.76	7.6	09.4 ± 0.05
2	8	Raw SPS	9.8	56.2	1.85	281	73.0	74.1 ± 2.28	10.1	97.8 ± 0.89
	9	Autoclaved SPS	9.3	55.8	1.73	269	69.1	74.3 ± 2.00	10.0	98.5 ± 0.17
	10	Purified SPS	11.4	53.5	1.56	329	61.3	81.3 ± 2.69	9.6	98.6 ± 0.41
	11	Raw CS	11.2	56.6	1.26	324	41.0	87.4 ± 0.52	10.2	99.4 ± 0.04
3	12	Low protein CS	2.9		1.26	83	24.8			
	13	Low protein SPS	2.6		2.58	74	54.2			

[1] Mean value of daily excrement per rat.

[2] Mean value ± standard deviation.

[3] CS = cornstarch; SPS = sweet potato starch; PS = potato starch.

termined by the method above described. Nitrogen content of the diet was determined by the Kjeldahl method and that in feces was determined by the micro-Kjeldahl method of A.O.A.C. ('50).

RESULTS

The results of this experiment are summarized in table 2. The digestibility of raw sweet potato starch was significantly lower than that of cornstarch, though the difference was less than 3%. Sixty per cent of raw potato starch was digested. Both raw sweet potato and raw potato starch decreased the

TABLE 3

Variance analysis of series 1

FACTOR	DEGREES OF FREEDOM	F-RATIO		
		Wt. of feces	Digestibility coeff.	
			Starch	Nitrogen
I[1]	7	1.6	1.8	0.6
M	2	536[2]	621[2]	243[2]
T	1	743[2]	834[2]	208[2]
I × M	14	0.7	1.8	2.4
I × T	7	0.8	1.5	3.2
M × T	2	528[2]	624[2]	221[2]
I × M × T	14	1	1	1

[1] I: individual difference, M: material, T: treatment.
[2] Significant at 1% level.

apparent digestibility of dietary protein. These effects of raw starches upon the digestibility of dietary starch and protein were not detected when the diets were steamed. Autoclaving of sweet potato starch in the dry state, however, showed little influence.

After the transformation of digestibility coefficient to arcsine and weight of feces to cubic root, the data of series 1 were analyzed statistically following the three-way layout method (Wilks, '44). The effects of material, heat treatment and individual difference and their interactions were studied. F-ratios of this variance analysis are given in table 3. The results of analysis show that factors of treatment, material

and interaction between material and treatment are significant at the 1% level. This means that the digestibility of dietary starch and that of protein vary according to the kind and the state of starch in the diet and that the effect of steaming varies according to the kind of starch. The treatment was most effective upon the potato starch diet and had little effect on the cornstarch diet.

The difference in digestibility between test 1 and test 7 was too small to be statistically significant. Since this result means that the seasonal difference in digestibility within the period of this experiment is negligible, the difference due to the order and the time of test may be left out of consideration.

Tables 4 and 5 show diagrams of the differences between the digestibility coefficients of the diets. Differences between raw starch diets were always significant but those between steamed diets were too small to be significant. The apparent digestibility of protein when fed with steamed sweet potato starch and potato starch was much improved. As shown in table 5, the digestibility of both starch and protein was inferior when sweet potato starch replaced corn starch. Nutrients in purified sweet potato starch diets, however, were digested better than those in raw sweet potato starch diet. Purified sweet potato starch seemed to take an intermediate place between raw cornstarch and raw sweet potato starch in nutritional value. There was no significant difference between autoclaved sweet potato starch and raw sweet potato starch. It was observed that raw sweet potato starch made very soft feces. Soon after the sweet potato starch diet was given, some rats had diarrhea for several days. Especially, when low protein sweet potato starch diet was fed, every rat suffered from severe diarrhea throughout the test period. That the weight of feces from rats on this diet was twice that of rats fed the low-protein cornstarch diet shows this fact clearly. The diarrhea disappeared as soon as diet was changed to the cornstarch diet. Raw potato starch made

TABLE 4

Diagram of difference between digestibility coefficients

(Results of series f)

DIET		RAW			STEAMED		
		CS	SFS	PS	CS	SPS	PS
		%	%	%	%	%	%
Raw	CS [1]		8.8 [2]	14.9 [2]	2.3 [2]		
Raw	SPS	2.5 [2]		6.1 [2]		7.4 [2]	
Raw	PS	42.1 [2]	39.6 [2]				12.5 [2]
Steamed	CS	0.4				0.9	0.1
Steamed	SPS		2.4 [2]	0.3			1.0
Steamed	PC		41.8 [2]	0.1	0.2		
		CS	SPS	PS	CS	SPS	PS
		RAW			STEAMED		

Protein / Starch

[1] CS = cornstarch; SPS = sweet potato starch; PS = potato starch.
[2] Significant at the 1% level.

TABLE 5

Diagram of difference between digestibility coefficients

(Results of series 2)

		CS	SPS		
		RAW	RAW	AUTOCLAVED	PURIFIED
		%	%	%	%
Raw	CS [1]		13.3 [2]	13.1 [2]	6.1 [2]
Raw	SPS	1.6 [2]		0.2	7.2 [2]
Autoclaved	SPS	0.9 [2]	0.7		7.0 [2]
Purified	SPS	0.8 [2]	0.8 [2]	0.1	
		RAW	RAW	AUTOCLAVED	PURIFIED
		CS	SPS		

Protein / Starch

[1] CS = cornstarch; SPS = sweet potato starch; PS = potato starch.
[2] Significant at the 1% level.

feces very large, white and sometimes caused diarrhea. This may be due to the excretion of much undigested starch.

Dietary and fecal starch contents determined by the direct hydrolysis method and by the method of Pucher et al. ('48) are compared in table 6. Rather small differences between the figures obtained by both methods are observed. Especially the agreement of dietary starch content is satisfactory. Very few reducing compounds other than starch are contained in the diet used.

TABLE 6

Comparison of starch intake, output and digestibility coefficient determined by direct hydrolysis (1) and by the method of Pucher et al. (2)

DIET		CONTENT IN THE DIET		DAILY INTAKE		DAILY OUTPUT		DIGESTIBILITY COEFF.	
		(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
		%	%	*gm*	*gm*	*mg*	*mg*	%	%
CS[1]	raw	50.9	51.1	7.6	7.7	53	2	99.3	99.97
	steamed	50.9	51.1	7.6	7.7	83	15	98.9	99.8
SPS	raw	50.1	51.2	7.5	7.7	242	85	96.8	98.9
	steamed	50.1	51.2	7.5	7.7	61	7	99.2	99.9
PS	raw	48.9	48.6	7.3	7.3	3144	2875	57.2	60.7
	steamed	48.9	48.6	7.3	7.3	71	9	99.0	99.9

[1] CS = cornstarch; SPS = sweet potato starch; PS = potato starch.

The digestibility of whole egg with raw cornstarch, calculated from the data of tests 11 and 12, is 95%, which agrees with the results of Mitchel and Bert ('54). As the rats had diarrhea, it may not be valid to assume the fecal nitrogen of test 13 as the endogenous value for rats fed the raw sweet potato starch diet. Therefore, true digestibility of whole egg with raw sweet potato starch is not known.

DISCUSSION

Coefficients of digestibility of starches shown in table 2 agree with those of Booher et al. ('51), whose coefficients were calculated from the content of nitrogen-free extract, not on direct measurements. It can be said that the direct hydrolytic

procedure is satisfactory for the analysis of starch contained in the diet and feces under the conditions of this experiment. In series 1, starch was also analyzed by the extraction method of Pucher et al. ('48). The result is compared with that obtained by the direct hydrolysis method in table 6. It seems reasonable to suppose that there exist in the feces fractions of starch which are not absorbed and which are so small that they do not precipitate with iodine and can not be determined by the method of Pucher et al. Therefore, the digestibility coefficient of starch based upon the data obtained by this method does not represent exactly the degree of utilization of dietary starch. The direct hydrolytic procedure always involves the risk that the figures obtained thereby may be higher than the true values on account of the possible existence of reducible materials other than sugars in the feces. Comparing those merits, the direct hydrolysis method was considered to be more satisfactory than for this specific determination and was used in series 2.

Cornstarch supported better utilization of dietary protein whether the protein was supplied as whole egg or a mixture of casein, fish meal and yeast. Both raw sweet potato starch and potato starch suppressed the utilization of dietary protein and they also caused diarrhea to a certain extent. However, the cause for this seems to be different for each starch. Forty-three per cent of dietary raw potato starch, 92% of which was in the form of starch large enough to precipitate with iodine, was excreted little attacked by digestive enzymes, in agreement with many previous workers' observations (Booher et al., '51; Jelinek et al., '52; Sakurai et al., '51). This may be explained by the digestion-resistant properties of the uppermost layer of the starch granule as Booher et al. ('51) suggested. The undigested starch granule, which will excite the intestinal canal, and which will swiftly pass through the digestive tract as Booher et al. ('51) observed, may be the main cause of diarrhea and of low utilization of protein in the potato starch diet. The digestion-resistance of potato starch disappeared on steaming. In the case of sweet potato

starch, on the contrary, fecal excretion of starch, determined by the method of Pucher et al. ('48), was very slight and this means that a very small quantity of sweet potato starch was excreted in the form of glucose chains long enough to precipitate with iodine. It is probable that some component of the sweet potato which is carried along with the starch caused diarrhea and that this in turn caused the decreased utilization of dietary protein, rather than that this is caused by the small quantity of digestion-resistant sweet potato starch residue. The finding that the purification process was effective in increasing the protein utilization and that the low-protein sweet potato starch diet (test 13) caused severe diarrhea without exception, adds support to the suggestion of the existence of some component which caused diarrhea. This component may combine with starch firmly, may be heat labile and rather water insoluble matter, and can be excluded by washing with alcohol, ether and sodium hydroxide solution.

SUMMARY

The digestibility of sweet potato starch and the degree of its effect upon the digestion of dietary protein were compared with those of cornstarch and potato starch, by feeding adult white rats diets containing one of these starches as the sole source of carbohydrate. The coefficients of digestibility of corn, sweet potato and potato starch were found to be 99, 97 and 57%, respectively. The differences are statistically significant. When the diets were steamed, all starches were utilized nearly perfectly and no significant differences were observed.

Raw sweet potato starch caused diarrhea and markedly decreased the apparent digestibility of dietary protein. This effect was decreased when sweet potato starch was purified, and disappeared when it was steamed. It is suggested that some component of the sweet potato which caused diarrhea is carried along with the starch, and that it would appear to be heat labile and can be removed by washing with certain solvents.

Raw potato starch was little utilized and it also lowered the apparent digestibility of the dietary protein. It is suggested that the main reason for this is the heat-labile digestion-resistant property of the potato starch granule.

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THE MICROBIOLOGICAL ASSAY OF THE AMINO ACIDS OF FIVE GENERA OF YEASTS GROWN UNDER CONTROLLED CONDITIONS[1,2]

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INTRODUCTION

Although many studies of the amino acid value of yeast proteins have been reported, most of them have been studies of brewers' yeast and have been confined to a limited number of amino acids (Stokes, Gunness, Dwyer and Caswell, '45; Horn, Jones and Blum, '50; Block and Bolling, '45; Edwards et al., '46). Only a few studies have been reported in which known genera of yeasts were investigated. Stokes and Gunness ('46) studied the amino acid composition of *Saccharomyces cerevisiae* and *Rhodotorula rubra* and Edwards and his co-workers ('46) reported on amino acid assays of *Torulopsis utilis*. This yeast has also been studied by the Lake States Yeast Corporation laboratories ('52) and data are presented for 14 amino acids. Kurth and Cheldelin ('46) reported 9 amino acids in *Mycotorula dipolytica, Hansenula suaveolens* and *Torulopsis utilis*. The purpose of the present

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study was to investigate the complete amino acid composition of the proteins of yeasts not previously reported.

METHODS

The yeasts used in this study were *Candida krusei* (NRRL-1736), *Pichia membranaefaciens* (ATCC 2254), *Saccharomyces carlsbergensis* (ATCC 9080) and *Hansenula anomala*

TABLE 1

Modified Wickerham's medium used for growing yeasts

(Amounts used per liter of medium)

	YEAST		
	H. anomala I	*H. anomala* II	*C. krusei; P. membranaefaciens; S. carlsbergensis*
Trace element solution [1]	10 ml	10 ml	10 ml
KH_2PO_4	1 gm	1 gm	1 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm	0.5 gm	0.5 gm
NaCl	0.1 gm	0.1 gm	0.1 gm
$CaCl_2 \cdot 2H_2O$	0.1 gm	0.1 gm	0.1 gm
Buffer solution [2]	100 ml	100 ml	100 ml
Glucose	20 gm	20 gm	100 gm
KNO_3	3.5 gm	...	...
$(NH_4)_2SO_4$	...	2.287 gm	2.287 gm
Biotin solution [3]	...	...	2 ml
Vitamin solution [4]	...	...	8 ml

[1] Trace element solution, micrograms per 10 ml: 500 H_3BO_3; 40 $CuSO_4 \cdot 5H_2O$; 100 KI; 200 $FeCl_3 \cdot 6H_2O$; 400 $MnSO_4 \cdot H_2O$; 200 $Na_2MoO_4 \cdot 2H_2O$; 400 $ZnSO_4 \cdot 7H_2O$.

[2] Buffer solution, grams per 100 ml: 1.54 citric acid; 1.391 Na_2HPO_4.

[3] Biotin solution: 100 μg per milliliter.

[4] Vitamin solution, micrograms per milliliter: 100 thiamine; 100 riboflavin; 200 nicotinic acid; 100 calcium pantothenate; 100 pyridoxine; 100 pyridoxal; 25 para aminobenzoic acid; 2 biotin; and 2 folic acid.

(NRRL Y365). The cells were grown in synthetic Wickerham's medium with glucose as the carbon source and ammonium sulphate as the nitrogen source. *Hansenula anomala* was also grown in a medium containing potassium nitrate as the nitrogen source. The exact compositions of the media are shown in table 1. The liquid medium was sterilized in three- or 6-liter florence flasks, each equipped with a gas

dispenser. Inoculum was added, and the flasks were incubated at room temperature with aeration for three or 4 days. It was necessary to add to the flask inoculated with *Saccharomyces carlsbergensis,* an anti-foaming agent, 2-ethyl-1-hexanol. When a luxuriant growth had been attained, the cells were removed from the liquor by centrifugation, washed three times, and dried to constant weight in a vacuum oven. For comparison, a sample of food grade debittered and washed brewers' yeast [5] from the brewery was assayed.

Duplicate samples of each of the 6 yeasts were hydrolyzed with 3 N HCl for 16 hours at 15 pounds pressure. After being approximately neutralized and made up to volume, the hydrolysates were filtered and stored in the refrigerator until used. For the tryptophan assays, duplicate samples of each yeast were hydrolyzed with barium hydroxide according to the method of Koch and Hanke ('48). All yeast hydrolysates were assayed for 18 amino acids using the microbiological method described by Schweigert, Guthneck, Kraybill and Greenwood ('49).

On the day of the assay, the amino acids were weighed and the assay medium was prepared from the stock solutions and these amino acids, omitting the one being assayed. On the same day a standard solution containing 200 μg per milliliter of the amino acid being studied was prepared. With an appropriately diluted solution, duplicate tubes were prepared using from 0 to 1.0 ml per tube in gradations of 0.1 ml. Two tubes were also prepared using 1.0 ml of the original concentrated standard amino acid solution. Duplicate assay tubes were prepared at levels of 0.2, 0.4, 0.6, 0.8, and 1.0 ml of yeast hydrolysate. Distilled water was added to bring the volume in each tube to 1.0 ml and finally 1.0 ml of the assay medium was added to each tube bringing the final volume to 2.0 ml. As duplicate samples of yeast hydrolysates were used, there were 4 assay tubes for each concentration of unknown. The media were sterilized, inoculated with appropriate test or-

[5] Kindly supplied by the St. Louis Brewers Yeast Corporation, 1514 North Thirteenth Street, St. Louis, Missouri, lot no. 021252.

ganisms and incubated for 72 hours at 30°C. After being removed from the incubator the fermentation acids were titrated immediately with 0.03 N NaOH or stored in the refrigerator prior to titration.

Average titration values of duplicate tubes agreeing within 10% of each other, at the 5 concentration levels of amino acid or yeast hydrolysate, were used for the calculations. Standard growth curves for the test organisms were considered valid when the blank was low, when the tube containing 1.0 ml of diluted amino acid solution approached maximum growth, and when the intermediate values formed a smooth curve with the steepest slope at the beginning of the curve. Amino acid values in yeast hydrolysates were determined from the standard growth curves. Assays were repeated when fewer than three values in each of the duplicate assays, 6 in all, were within a 10% range. The figures reported are the average of all the values within this range.

Test organisms were maintained on stock agar stabs and transferred weekly. The day preceding the assay, 10 ml of the sterile culture medium without agar was inoculated from the stock culture. After incubation for 24 hours the cells were washed and suspended in sterile saline solution. This was used as the inoculum for the assay tubes. *Lactobacillus arabinosus* 17–5, [*Streptococcus equinus* (McClesky, '52)] was used for the assay of glutamic acid, isoleucine, leucine, tyrosine and valine. *Leuconostoc citrovorum* [*Pediococcus cerevisiae* (Felton and Niven, '53)] was used for alanine and *Streptococcus faecalis* R for arginine, methionine, threonine and tryptophan. For all other amino acids *Leuconostoc mesenteroides* was used. These included aspartic acid, cystine, glycine, histidine, lysine, phenylalanine, proline and serine.

The nitrogen value of the yeasts was determined by the method of Willits and Ogg ('50) with slight modifications. All samples were analyzed in quadruplicate with duplicate blanks for each test. Results that varied more than 3% were discarded.

RESULTS AND DISCUSSION

The nitrogen values of the yeasts studied are shown in table 2. The value obtained for brewers' yeast, 8.76%, is similar to values reported by other workers (Reisen, Schweigert and Elvehjem, '46; Horn, Jones and Blum, '50; Stokes, Gunness, Dwyer and Caswell, '45; Gunness, Dwyer and Stokes, '46). The nitrogen values of the yeasts grown under the conditions described above were somewhat lower. This may be due to species differences or possibly to different growth media.

TABLE 2

Total nitrogen and crude protein in five genera of yeasts

NAME OF YEAST	NO. SAMPLES ANALYZED	% N			PROTEIN, GM PER 100 GM YEAST [1]
		Range	Av.	% Diff.	
H. anomala I	4	6.69–6.88	6.81	2.84	42.6
H. anomala II	4	6.82–6.99	6.88	2.40	43.0
C. krusei	3	7.36–7.55	7.45	2.58	46.6
P. membranaefaciens	3	5.53–5.62	5.57	1.63	34.8
S. carlsbergensis	3	7.15–7.32	7.23	2.38	45.2
Brewers' yeast	4	8.73–8.77	8.76	0.46	54.8

[1] Calculated as N × 6.25.

If protein of yeast is assumed to be 16% nitrogen, the crude protein found in the yeasts studied ranged from 34.8% of the dry weight of *Pichia membranaefaciens* to 54.8% of brewers' yeast. Although this assumption provides a simple method of treating the data so that comparisons may be made, it should be considered only an approximation because yeast is known to contain relatively large amounts of nucleic acids and other nitrogenous materials. Carter and Phillips ('44), in a review of early work on the nutritive value of yeast, concluded that protein figures derived in this way (N × 6.25) are 12 to 18% too high because of the large amount of non-protein nitrogen found in yeasts. Their conclusion was based on reports of Meisenheimer ('21) that purines account for 8 to 13% of the total nitrogen, pyrimidines for 4%, choline and glucosamine for 0.5% each; and reports

by von Soden and Dirr ('42) that only 80% of the total nitrogen of yeast is protein nitrogen. In a more recent review, Carter ('48) reports that Dirr and Decker ('44) subsequently found only 65 to 76% of the total nitrogen of yeast to be protein nitrogen. The amino acids found in the present study account for 73 to 79% of the total nitrogen found in the various yeasts.

TABLE 3

Amino acid values of yeast

(grams per 100 gm)

AMINO ACIDS	*H. anom.* I	*H. anom.* II.	*Candida krusei*	*Pichia membr.*	*Sach. carlsb.*	BREWERS' YEAST
Alanine	2.5	1.9	1.9	1.4	1.9	2.7
Arginine	2.1	1.9	2.1	1.5	2.0	3.0
Aspartic acid	5.2	5.5	6.0	4.2	5.7	6.8
Cystine	0.04	0.05	..	..	..	0.08
Glutamic acid	4.7	6.0	5.0	4.5	6.5	6.4
Glycine	2.0	2.4	2.1	1.7	2.1	2.6
Histidine	0.7	0.7	0.9	0.6	1.0	1.0
Isoleucine	1.8	2.5	2.2	1.9	2.0	2.1
Leucine	3.0	3.7	3.5	2.8	3.3	3.7
Lysine	3.3	3.6	3.7	2.7	3.7	4.5
Methionine	0.3	0.4	0.8	0.6	0.5	0.6
Phenylalanine	1.6	2.0	2.0	1.5	1.8	2.1
Proline	1.7	1.9	1.7	1.2	1.7	2.1
Serine	1.2	1.2	1.2	0.8	1.3	1.3
Threonine	1.9	2.1	2.3	1.7	2.1	2.1
Tryptophan	0.5	0.5	0.5	0.4	0.5	0.6
Tyrosine	1.4	1.9	1.8	1.3	1.7	1.8
Valine	2.4	2.8	2.7	2.1	2.5	2.9
Total N	6.81	6.88	7.45	5.57	7.23	8.76

The quantities of amino acids found in the yeasts are shown in tables 3 and 4. Amino acid values of yeasts reported in recent literature, most of which were obtained by microbiological assay, are shown for comparison in tables 5 and 6. It will be seen that although there are many differences in the amino acid composition of various yeasts there are also striking similarities. All yeast proteins contain the 8 so-

called nutritionally essential amino acids, making them potential supplements of poor quality proteins. All are relatively good sources of lysine; all are relatively poor sources of cystine and methionine.

Although the alanine, arginine, aspartic acid, glutamic acid and other amino acids of these yeasts contribute to the nutritive value of their proteins, special comment will be made

TABLE 4

Amino acids in yeast protein

(% of protein [N × 6.25])

AMINO ACIDS	*H. anom.* I	*H. anom.* II	*C. krusei*	*P. membr.*	*S. carlsb.*	BREWERS' YEAST
Alanine	5.9	4.3	4.0	4.1	4.2	5.0
Arginine	5.0	4.4	4.4	4.2	4.4	5.4
Aspartic acid	12.2	12.7	12.8	12.0	12.6	12.3
Cystine	0.1	0.1	...	...	...	0.1
Glutamic acid	10.9	13.9	10.8	13.0	14.4	11.6
Glycine	4.6	5.5	4.5	4.7	4.6	4.8
Histidine	1.6	1.7	1.9	1.8	2.1	1.8
Isoleucine	4.2	5.8	4.7	5.5	4.4	3.8
Leucine	7.1	8.6	7.5	8.0	7.2	6.8
Lysine	7.7	8.4	7.9	7.6	8.2	8.2
Methionine	0.7	0.9	1.8	1.7	1.2	1.1
Phenylalanine	3.8	4.7	4.3	4.3	4.0	3.8
Proline	4.0	4.3	3.6	3.6	3.7	3.8
Serine	2.8	2.8	2.6	2.3	2.9	2.4
Threonine	4.5	4.9	4.9	4.9	4.6	3.8
Tryptophan	1.2	1.2	1.1	1.1	1.1	1.1
Tyrosine	3.4	4.4	3.9	3.7	3.7	3.3
Valine	5.6	6.5	5.8	6.0	5.5	5.3
Total N, %	6.81	6.88	7.45	5.57	7.23	8.76

only about the 8 essential amino acids, and comparisons will be made with the amino acid values of other foods (Horn, Jones and Blum, '50). Isoleucine values of the yeasts examined in this study are quite similar. *Hansenula anomala* II, i.e., cells grown on ammonium sulphate medium, and *Pichia membranaefaciens* are the best sources of this amino acid with values well above that obtained for brewers' yeast. The

isoleucine levels of these two yeasts are similar to those of milk and beef proteins, which are considerably higher than the isoleucine values of vegetable proteins. The leucine values of all yeasts are well below that of milk and egg protein and similar to most vegetable proteins. The lysine content of all the yeasts studied is similar to that of the brewers' yeast, milk and egg protein. All of these are richer sources of lysine than are vegetable proteins.

TABLE 5

Amino acid values of yeasts reported in the literature

(grams per 100 gm)

AMINO ACIDS	*Saccharomyces cerevisiae* [1]	*Rhodotorula rubra* [2]	*Mycotorula dipolytica* [3]	*Hansenula suaveolens* [3]	*Torulopsis utilis* [3]	BREWERS' YEAST [4]	BREWERS' YEAST [5]
Arginine	2.4	3.7	3.2	2.9	3.1	2.6	2.26
Histidine	2.07	1.99	1.4	1.4	1.5	1.2	0.78
Isoleucine	2.5	2.1	3.5	3.7	3.7	2.4	2.42
Leucine	3.8	3.3	3.7	3.6	3.8	4.1	3.25
Lysine	3.1	3.0	4.4	4.3	4.4	3.7	3.5
Methionine	0.65	0.53	..	..	..	0.79	0.53
Phenylalanine	2.1	1.72	2.4	2.4	2.3	2.5	1.79
Threonine	2.4	1.79	2.5	2.4	2.5	2.9	2.54
Tryptophan	0.59	0.45	0.3	0.3	0.3	0.6	..
Valine	2.8	2.5	3.1	3.3	3.3	3.1	2.54
Total N, %	8.94	8.95	..	..	..	9.14	7.71

[1] Reisen, Schweigert and Elvehjem, '46.
[2] Stokes and Gunness, '46.
[3] Kurth and Cheldelin, '46.
[4] Stokes, Gunness, Dwyer and Caswell, '45.
[5] Horn, Jones and Blum, '50.

The amount of methionine determined in this study for brewers' yeast is similar to that reported by other workers (table 5). Among the yeasts studied, *Candida krusei* is the best source of methionine, 1.8%. In evaluating the methionine value of a protein, cystine values should also be taken into consideration. Yeast protein is known to be deficient in both of these sulphur-containing amino acids. Results obtained for cystine in this study are below those obtained by earlier

TABLE 6

Amino acids in yeast protein reported in the literature

(% of protein [N × 6.25])

AMINO ACIDS	*Saccharomyces cerevisiae* [1]	*Rhodotorula rubra* [1]	TORULA YEAST [2]	*Torulopsis utilis* [3]	"KITCHEN FOOD YST." [3]	BREWERS' YEAST				
						[4]	[5]	[6]	[2]	[3]
Arginine	4.3	6.6	4.15	8.6	7.0	4.5	4.3	4.7	4.0	13.1
Cystine	..		1.0	..	..	..	1.0	..	..	..
Glutamic acid	..	..	13.37	..	..	..	..	..	14.0	..
Glycine	..	..	4.54	..	..	..	..	..	4.2	..
Histidine	3.7	3.6	1.71	2.8	2.84	2.1	2.8	1.6	2.0	3.0
Isoleucine	4.5	3.8	5.51	5.5	5.70	4.2	5.9	4.9	5.3	6.0
Leucine	6.8	5.9	7.57	8.3	6.8	7.1	7.4	6.7	7.0	7.8
Lysine	5.5	5.4	6.87	6.84	6.34	6.4	7.5	7.3	6.9	7.4
Methionine	1.2	0.95	0.83	2.62	6.42	1.37	2.7	1.1	1.3	2.34
Phenylalanine	3.8	3.1	3.86	3.59	4.09	4.4	4.1	3.7	3.8	3.59
Threonine	4.3	3.2	5.42	5.07	4.88	5.1	5.5	5.3	5.1	5.07
Tryptophan	1.1	0.8	1.65	1.63	3.22	1.05	1.3	0.9	1.5	1.63
Tyrosine	..	..	15.6	..	..	..	3.6	..	..	..
Valine	5.0	4.5	6.08	6.40	5.90	5.4	5.0	5.3	5.9	6.40
Total N, %	8.94	8.95				9.14		7.7		

[1] Stokes and Gunness, '46.
[2] C. N. Frey quoted by Carter, '48.
[3] Edwards et al., '46. Brewers' yeast, blend of three yeasts.
[4] Stokes et al., '45.
[5] Block and Bolling, '45. Average, 8 yeasts.
[6] Horn, Jones and Blum, '50.

workers and probably do not represent the true cystine content of these yeasts. Autoclaving temperatures are known to destroy a large part of the cystine present in a sample (Reisen, Spengler, Robblee, Hanks and Elvehjem, '47). It is probable that the figures presented should be considered as approximate and taken as an indication that these yeast proteins contain very little cystine.

The phenylalanine values for all the yeasts studied are similar to those previously reported for other yeasts and to milk, meat and vegetable proteins. Threonine values of the proteins of yeasts grown in this laboratory are all above the values for brewers' yeast. However, they are comparable to amounts found by other workers (table 6). The tryptophan levels are similar to those reported from other laboratories. They are also similar to values reported for milk and meat proteins, but below egg proteins. Valine values for all yeasts studied are similar to those previously reported. In this study *Hansenula anomala* II contained the greatest amount of valine. Yeast proteins as a source of this essential amino acid are exceeded only slightly by milk and egg proteins.

Yeast protein is equal or superior to most vegetable proteins with respect to the quantities of all the essential amino acids it contains, and it compares favorably with good quality animal proteins except in methionine. If it is ever to serve as a substitute for animal protein the methionine content must be increased either by finding a strain of yeast that produces more methionine or by encouraging its production through changes in the growth medium. This problem has been studied by Chiao and Peterson ('53), who found that changes in the growth medium could increase both the amount of protein and the amount of methionine produced by a given species, but that the methionine value of the protein remained constant. Of 20 species studied by these workers, methionine, expressed as per cent of dry weight of yeast, varied from 0.17% for *Endomycopsis fubuliger* to 1.0% for *Rhodotorula gracilis*.

In the present study *Hansenula anomala* was grown on both nitrate and ammonium media. Although the total nitrogen of these two samples was the same, 6.81 and 6.88% respectively, the amino acid compositions were quite different. The yeast grown with ammonium sulphate contained more glutamic acid, glycine, isoleucine, leucine, phenylalanine, tyrosine and valine and less alanine and arginine than the same yeast grown on nitrate medium, although the growing conditions and other components of the media were identical.

SUMMARY

The complete amino acid composition of the proteins of 4 genera of yeasts not previously reported were determined by the microbiological assay method. The yeasts studied, *Candida krusei, Pichia membranaefaciens, Saccharomyces carlsbergensis,* and *Hansenula anomala,* were grown under standardized conditions in synthetic Wickerham's medium containing glucose and ammonium sulphate. *Hansenula anomala* was also grown on identical medium containing potassium nitrate. Brewers' yeast was assayed for comparison, and several amino acids are reported herewith for the first time.

The total nitrogen of the yeasts varied from 5.57% in *Pichia membranaefaciens* to 7.45% in *Candida krusei,* somewhat lower than the brewers' yeast value of 8.76%. Amino acids accounted for 73 to 79% of the total nitrogen in the various yeasts. All yeasts studied contain the 8 so-called nutritionally essential amino acids making them potential supplements of poor quality proteins. All are relatively good sources of lysine; all are relatively poor sources of cystine and methionine. *Hansenula anomala* grown in the ammonium sulphate medium contained the same amount of protein as that grown in the potassium nitrate medium but the former contained more glutamic acid, glycine, isoleucine, leucine, phenylalanine, tyrosine, and valine and less alanine and arginine than the latter, although the growing conditions and other components of the media were identical.

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STUDIES ON THE UTILIZATION OF DIETARY ISOLEUCINE BY THE GROWING ALBINO RAT

I. ISOLEUCINE REQUIREMENTS DETERMINED WITH AMINO ACID MIXTURES

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The requirement of the growing albino rat for dietary L-isoleucine has been investigated by Rose et al. and by Hegsted et al. Rose et al. ('49) reported that 0.50% of L-isoleucine of the diet is the minimum amount consistent with maximum growth and (Rose et al., '48) that amino acid mixtures containing nitrogen equivalent to 12.5% of conventional protein are required for maximum growth. Thus, under these conditions, one is led to conclude that the rat requires 4.0% of L-isoleucine expressed as a percentage of the dietary protein. Hegsted et al. ('45), using human serum albumin at 20% of the diet, reported that the total L-isoleucine requirement of the growing rat is 0.78% of the diet, or 3.9% of the protein. These results support the view that L-isoleucine requirements may be best expressed as a percentage of the dietary protein.

The following experiment was designed to investigate further the L-isoleucine requirement as affected by nitrogen concentration, employing exogenous urinary nitrogen excretion as a percentage of nitrogen intake as the criterion of L-isoleucine adequacy of the diet.

PROCEDURE

Twenty-eight weanling male albino rats of Sprague-Dawley strain were individually fed ground stock ration up to 6 gm daily for a three-day preliminary period. They were then allotted at random for feeding the test diets. Throughout the experiment food intake was restricted to 6 gm of diet per day. After the assignment of rats to position in the experiment, a 4-day period of adjustment to the amino acid-containing diets was employed during which time all rats received L-isoleucine at a level of 2.5% of the amino acid mix. The first of three contiguous experimental periods followed this adjustment period. Each period consisted of 6 days, urine being collected separately from each rat for the last 4 days of each period, using 50 ml of a solution containing 2% of copper sulfate and 1% of sodium fluoride to preserve the urine. The diets, shown in table 1, were designed to be complete except for isoleucine. The amino acid mix was incorporated so as to provide 10 or 18% of the diet. Seven levels of isoleucine supplementation to each level of amino acid mix were obtained by the addition of appropriate amounts of a solution containing 24 mg of DL-isoleucine per milliliter to each diet at the time of feeding. Levels of isoleucine supplementation were chosen to be equivalent to 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5% of L-isoleucine added to the amino acid mixture. Total nitrogen was determined on the mixed diets by macro-kjeldahl, and on the collected urine by micro-kjeldahl analysis.

The basic design was a Youden square, each isoleucine level occurring in each of the three experimental periods, each of 7 rats receiving a different isoleucine level in each period, and each isoleucine level occurring once on the same rat with each other level. Two such squares were used for each level of amino acid mix. The assignment of rats to sets of isoleucine levels and levels of amino acid mix was randomized. A Youden square of the kind used appears in table 2, rats being designated by number and isoleucine levels by letter.

TABLE 1

Composition of basal diets

INGREDIENT	10% AMINO ACID [1] DIET	18% AMINO ACID [2] DIET
	%	%
Amino acids [3]		
Non-essential	5.13	9.25
Essential	4.87	8.76
Salts 446 [4]	4.00	4.00
NaCl	1.00	1.00
$NaHCO_3$	0.80	1.40
Vitamin mix 691 [5]	5.00	5.00
Cod-liver oil	1.50	1.50
Wheat germ oil	0.50	0.50
Corn oil	15.00	15.00
Roughage [6]	2.00	2.00
Sucrose	30.10	25.80
Starch	30.10	25.80

[1] By analysis contained nitrogen equivalent to 8.49% conventional protein.

[2] By analysis contained nitrogen equivalent to 15.30% conventional protein.

[3] Contained amino acids as follows, expressed as percentage of the conventional protein equivalent: glutamic acid 14.80, DL-serine 3.74, glycine 5.21, L-tyrosine 10.45, L-cystine 1.48, L-proline 6.71, L-asparagine 9.10, DL-alanine 8.95, L-lysine HCl 8.15, L-arginine HCl 4.92, DL-tryptophan 1.31, DL-phenylalanine 5.90, DL-leucine 10.50, DL-threonine 6.55, DL-valine 9.15, DL-methionine 3.86, DL-histidine $HCl \cdot H_2O$ 7.10.

[4] Spector ('43).

[5] Forbes and Vaughan ('54).

[6] Woodflock, obtained through the Brown Company, Portland, Maine.

TABLE 2

Design of experiment

RAT	1	2	3	4	5	6	7
Trial 1	A	B	C	D	E	F	G
Trial 2	B	C	D	E	F	G	A
Trial 3	D	E	F	G	A	B	C

RESULTS

The data on urinary nitrogen excretion were corrected for endogenous nitrogen (based on estimated body surface area) and, as finally used in calculations, represent the exogenous nitrogen excreted daily expressed as a percentage of the nitrogen intake. These values are given in table 3.

The data reveal a highly significant ($P < 0.01$) reduction in error by correcting nitrogen excretion for variable food intake, a highly significant ($P < 0.01$) effect of amino acid mix level, both before and after adjustment for food intake, and of isoleucine level before adjustment, and a significant ($P < 0.05$) effect of isoleucine level after adjustment but no interaction between level of amino acid mix and level of isoleucine.

TABLE 3

Exogenous nitrogen extreted daily, expressed as percentage of total nitrogen intake

L-ISOLEUCINE (% OF CONVENTIONAL PROTEIN EQUIVALENT)	8.49% CONVENTIONAL PROTEIN EQUIVALENT RATION						
	1.76	2.35	2.94	3.53	4.12	4.71	5.30
Period 1	61.2	44.0	31.9	26.5	37.0	27.1	35.8
	44.0	42.3	31.5	28.0	40.9	26.6	31.9
2	48.5	35.3	36.0	26.0	32.2	25.0	30.7
	61.0	54.2	29.5	24.0	30.8	31.5	30.8
3	58.2	43.2	34.3	25.7	24.7	27.7	26.0
	61.5	40.2	33.6	41.6	26.8	35.0	35.0
	15.30% CONVENTIONAL PROTEIN EQUIVALENT RATION						
Period 1	76.5	50.8	27.6	42.0	38.0	40.2	38.0
	64.5	43.7	39.5	35.3	54.3	36.2	35.0
2	74.3	48.5	44.3	47.5	36.0	37.0	39.7
	70.6	59.2	44.7	43.3	40.2	35.8	39.0
3	56.7	48.5	52.0	43.5	44.3	47.7	41.2
	57.0	54.0	51.0	45.5	36.8	45.1	48.0

The bending point of the bent line (falling straight, then horizontal) representing nitrogen excretion as related to L-isoleucine level, estimated by least squares, was found to be at a level of 2.2% of the amino acid mix, or 2.6% of the conventional protein equivalent of the diet. This value was independent of the level of conventional protein equivalent of the diets, 8.49 and 15.30%.

DISCUSSION

As stated previously, the food intake of each rat was limited to 6.0 gm daily. However, animals receiving the two lower levels of isoleucine supplementation consumed an average of

4.3 and 4.9 gm/day. Hence nitrogen excretion was corrected for variable food intake, reducing the error variance from 10.14 to 5.53, with 23 degrees of freedom.

The minimum level of L-isoleucine found in this experiment to be required for minimum nitrogen loss in the urine is considerably lower than the 4.0% level derived from Rose's data and reported by Hegsted to be required for maximum weight gain. Several explanations may be offered for this difference. The present study attempted to find the point to which L-isoleucine might be lowered without inhibiting the efficiency with which the dietary protein was used. This may differ from the level required to produce maximum weight gain under those ad libitum feeding conditions which are not designed to study efficiency of utilization but only the over-all effects of consuming variable amounts of rations of different nutritive value. Hegsted's investigation was carried out using a natural protein as the major nitrogen source, and while his technique was similar to Rose's, a further complication is the possibility that the isoleucine in human serum albumin preparation was not completely available to rats. The results of a series of investigations conducted in this laboratory to investigate this latter point are being prepared for publication and seem to support this interpretation.

SUMMARY

The minimum amount of L-isoleucine required to promote minimum wastage of dietary nitrogen by growing albino rats fed diets whose nitrogen was supplied as amino acids amounting to 8.49 and 15.30% conventional protein equivalent was found to be 2.6% of the conventional protein equivalent of the diets, irrespective of the total nitrogen content of the diet.

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OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1956 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1956. For details of nomination procedure, write to Chairman of the Nominating Committee.

Chairman, Nominating Committee:
DR. OTTO A. BESSEY
Department of Biochemistry and Nutrition
University of Texas Medical Branch
Galveston, Texas

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Nominations are solicited for the 1956 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers during the previous calendar year, but the Jury of Award may recommend that it be given for important contributions made over a more extended period of time not necessarily including the previous calendar year. The award is usually given to one person, but if in their judgment circumstances and justice so dictate, the Jury of Award may recommend that it be divided between two or more collaborators in a given research. The Jury may also recommend that the award be omitted in any given year if in its opinion the work submitted does not warrant the award. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1956. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1956. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award. For details of nomination procedure, write to Chairman of the Nominating Committee.

Chairman, Nominating Committee:
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