Biopharmacology of Anabolic Agents II

Radio-Phosphorus Uptake in Molars of Rats Fed Norethandrolone and Phosphorus-Enriched Caries-Test Diets

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No statistically significant difference existed between the radio-phosphorus content in the molar teeth of animals treated with norethandrolone and the respective control animals. In addition, radio-phosphorus levels in the molar teeth of norethandrolone-treated animals fed a high phosphorus diet equalled the radio-phosphorus level in those animals on a high phosphorus diet alone. These results were not effected by the age of the animal within the experimental period studied, nor was there a difference in these results upon comparison of first, second, and third molar teeth with their respective controls.

Considerable experimental evidence concerning the cariostatic effect of phosphorus may be found in the literature. For example, a very pronounced cariostatic effect has been obtained with supplements of dibasic sodium phosphate (1–9). Previous experiments in our laboratories (10) indicated that when 2% disodium phosphate is added to a caries-test diet, Sprague-Dawley strain albino rats have less tooth decay in molar teeth than the respective controls.

The addition of norethandrolone to the caries-test diet fed Sprague-Dawley albino rats increased the incidence of molar tooth decay over that found in animals fed the basic caries-test diet alone. When 2% disodium phosphate was added to the diet of norethandrolone-treated animals, the caries incidence was decreased. The incidence approximated that of control animals fed the caries-test diet supplemented with 2% disodium phosphate. Also, the addition of 2% disodium phosphate to the diet of norethandrolone-treated animals considerably reduced the caries incidence in molar teeth when compared to norethandrolone-treated animals fed the basic caries-test diet only. Thus, it would appear that the addition of 2% disodium phosphate to the diet caused a reversal of norethandrolone cariogenesis under the experimental conditions employed.

According to Solomons, Irving, and Neuman (11), and others, the organic matrix of dentin consists mainly of collagen, with small amounts of mucopolysaccharides similar to chondroitin sulfate. They found that freshly decalcified human dentin could induce formation of hydroxyapatite from calcium-phosphorus solutions. Also, Takuma (12) observed the regular deposition of crystals on the matrix fibrils in human dentin. Therefore, the fibrous protein of dentin and enamel plays a significant role in the deposition and growth of apatite crystals.

Norethandrolone has been shown to exert an effect on the synthesis of chondroiten sulfate and collagenous tissues (13). In addition, upon clinical testing, norethandrolone has been found to increase phosphorus retention (14–16). Since norethandrolone has an effect on phosphorus retention and collagen synthesis, a study of the influence of norethandrolone on phosphorus deposition in rat molar swas undertaken. In an investigation of this type radiological techniques utilizing radio-phosphorus seemed to be the logical procedure.

EXPERIMENTAL

A total of 192 weanling Sprague-Dawley strain albino rats, weighing from 45–50 Gm. each, was divided randomly into four groups of 48 animals each (24 females and 24 males). Sexes were separated and the animals housed three to a cage on screenwire bottoms. All animals were given concentrated vitamin A, D, and E supplement by mouth weekly and distilled water *ad libitum*. All rats were

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TABLE I.—STATISTICAL RELATIONS OF RADIO-PHOSPHORUS CONTENT IN MOLAR TEETH, $P = 0.05$

Time Interval	Male Relations	Female Relations	Male Relations	Female Relations
	Paired Lower First Molar		Paired Upper First Molar	
2	NP[a] = P[b]	NP = P	NP = P	NP = P
2	N[c] = C[d]	N = C	N = C	N = C
4	NP $>$ P	NP = P	NP $>$ P	NP = P
4	N = C	N = C	N = C	N = C
6	NP = P	NP = P	NP = P	NP = P
6	N = C	N = C	N = C	N = C
8	NP = P	NP = P	NP = P	NP = P
8	N = C	N = C	N = C	N = C
	Paired Lower Second Molar		Paired Upper Second Molar	
2	NP = P	NP = P	NP = P	NP = P
2	N = C	N = C	N = C	N = C
4	NP = P	NP = P	NP $>$ P	NP = P
4	N = C	N = C	N = C	N = C
6	NP = P	NP = P	NP = P	NP = P
6	N = C	N = C	N = C	N = C
8	NP = P	NP = P	NP = P	NP = P
8	N = C	N = C	N = C	N = C
	Paired Lower Third Molar		Paired Upper Third Molar	
2	NP = P	NP = P	NP = P	NP = P
2	N = C	N = C	N $<$ C	N = C
4	NP = P	NP = P	NP = P	NP = P
4	N = C	N = C	N = C	N = C
6	NP = P	NP = P	NP = P	NP $>$ P
6	N = C	N = C	N = C	N = C
8	NP = P	NP = P	NP = P	NP = P
8	N = C	N = C	N = C	N = C

[a] NP (Group 1) = norethandrolone plus 2% Na_2HPO_4 added to diet. [b] P (Group 2) = 2% Na_2HPO_4 added to diet. [c] N (Group 3) = norethandrolone added to diet. [d] C (Group 4) = basic diet.

weighed twice weekly. Animal cages were washed with soap and water, rinsed and dried three times a week, while the special feeding cups were washed each morning. All animals were injected intraperitoneally once a week with radio-phosphorus, with the dosage based on 2 μc. per 100 Gm. of body weight. The solutions for injection were prepared from a stock solution of $H_3P^{32}O_4$ in weak HCl by diluting, volumetrically, measured aliquots with 0.001 *M* sodium phosphate to desired volumes. Radio-phosphorus concentrations were prepared not to exceed a volume of 1 ml. per injection per animal.

Animals were given identically weighed quantities of diet with each group fed variations of the basic caries-test diet (Stephan Diet 580)[1] as follows: Group 1 was fed the basic diet to which was added 2% Na_2HPO_4 and norethandrolone, Group 2 was fed the basic diet with added 2% Na_2HPO_4, Group 3 was fed the basic diet with added norethandrolone, and Group 4 was fed the basic diet only.

When 2% Na_2HPO_4 was added to the diet, the salt replaced an equal amount of cane sugar. An analysis of the caries-test diet was obtained to determine the content of phosphorus in the basic diet compared to that in the basic diet plus added Na_2HPO_4. The basic diet contained 0.36% phosphorus, or about half the phosphorus found in the basic diet supplemented with Na_2HPO_4 (0.70% phosphorus). Thus, the animals subsisting on basic diet received considerably less phosphorus than those animals fed the basic diet with added Na_2HPO_4.

Those animals being treated with norethandrolone received a dose closely approximating 5 mg./Kg. of body weight per day during the entire 56-day experimental period. This was accomplished by thoroughly incorporating the drug into the basic caries-test diet. The amount of drug incorporated was based on the average daily weight of the animals in each treated group. A weighed quantity of this diet was placed in each special feeder for those animals receiving the drug sufficient for daily requirements. The following morning refused food was discarded after weighing. Dosage calculations were based on food consumed per day by each cage of three animals over the 56-day period.

At the end of 2 weeks, 48 animals (representing six animals of each sex from each experimental group) were sacrificed with chloroform. Bilateral extractions of the maxillary and mandibular molars were performed. The upper jaw (left and right), first molar teeth were paired and weighed with a model H-5 gram-atic mettler balance. The teeth were dissolved in approximately 1 ml. of nitric acid, with the aid of infrared heat lamps, in a pyrex glass planchet 1 in. in diameter and $^5/_{16}$ in. in depth. This procedure was followed for upper jaw second and third molars, as well as for all lower jaw molars. The samples were dried with infrared heat lamps, the radioactivity determined and corrected for decay.

Determination of radioactivity was accomplished with Tracerlab "1000" scalers connected to shielded end-window geiger tubes. Each sample was counted five times, with sufficient time allowed to accumulate at least 500 counts per determination, thus incurring a probable error of approximately 3%. Additional groups of 48 animals, distributed as in the 2-week study, were sacrificed at 4, 6, and 8-week intervals. The animals were treated in the

[1] Stephan Diet 580: skim milk powder, 32%; cane sugar, 66%; whole dried liver substance N.F., 2%.

TABLE II.—RADIO-PHOSPHORUS LOCATED IN THE MOLAR TEETH OF MALE RATS

		Av. c.p.m./mg. ± S.D.					
Animals	Group	Lower First	Lower Second	Lower Third	Upper First	Upper Second	Upper Third
			2-Wk. Interval				
6	1[a]	15.0	15.1	43.3	13.1	14.3	40.7
		±1.74	±1.09	±4.96	±1.92	±1.86	±3.08
6	2[b]	12.8	13.5	40.2	12.6	13.2	39.9
		±2.85	±3.66	±9.53	±3.93	±3.74	±10.1
6	3[c]	20.5	21.2	59.6	20.2	19.2	55.0
		±5.29	±0.95	±7.58	±2.86	±3.50	±10.7
6	4[d]	22.8	20.9	62.7	21.5	21.1	67.9
		±1.11	±4.70	±4.45	±4.87	±5.71	±6.83
			4-Wk. Interval				
5	1	16.1	16.8	30.7	15.9	15.1	31.0
		±1.66	±3.85	±0.81	±0.83	±1.49	±0.69
6	2	13.6	13.1	27.5	13.1	12.6	27.6
		±1.34	±1.44	±2.42	±1.14	±1.64	±3.71
4	3	21.8	21.2	41.8	20.9	17.7	41.0
		±3.06	±1.28	±4.67	±2.65	±0.83	±4.94
5	4	22.7	20.7	43.1	21.0	20.4	42.8
		±1.23	±1.97	±4.27	±2.28	±2.55	±5.06
			6-Wk. Interval				
5	1	17.1	15.8	24.3	15.9	15.8	23.8
		±2.03	±2.18	±2.00	±1.97	±2.13	±2.50
5	2	15.1	14.8	24.5	15.0	14.4	23.5
		±0.86	±1.35	±1.17	±0.74	±1.96	±1.88
6	3	25.7	24.0	34.7	22.3	20.8	33.9
		±3.87	±3.04	±6.81	±3.01	±2.86	±5.99
6	4	22.3	25.2	35.8	24.1	22.6	34.8
		±4.41	±5.26	±3.39	±2.13	±0.97	±3.49
			8-Wk. Interval				
6	1	13.2	13.1	17.1	13.0	12.2	17.4
		±1.24	±1.09	±0.57	±1.70	±1.05	±0.78
6	2	13.9	14.3	17.4	13.1	11.9	16.6
		±1.69	±3.29	±1.44	±0.95	±0.99	±0.80
6	3	23.9	22.6	29.4	21.7	20.4	24.4
		±1.73	±2.11	±2.26	±2.58	±1.46	±5.62
6	4	21.8	21.0	27.4	20.8	19.9	24.2
		±2.87	±2.85	±2.83	±3.15	±2.96	±6.80

[a] Group 1 = norethandrolone plus 2% Na_2HPO_4 added to diet. [b] Group 2 = 2% Na_2HPO_4 added to diet. [c] Group 3 = norethandrolone added to diet. [d] Group 4 = basic diet.

same manner as those rats killed after 2 weeks of experimentation.

One male animal died in each of the following groups: Group 1 and 4 of the 4-week time interval, and Group 1 and 2 of the 6-week time interval. Two male animals died in the Group 3 of the 4-week study.

RESULTS AND DISCUSSION

Table I shows the relation of the radio-phosphorus content in the various male and female molar teeth based on statistical comparisons. Tables II and III show the radio-phosphorus located in the molar teeth of male and female rats, respectively.

It would seem that, under the conditions of this experiment, norethandrolone had no effect on radio-phosphorus deposition in rat molars in animals on high or low phosphorus caries-test diets compared with their respective controls. Table I shows that the radio-phosphorus levels in the norethandrolone-treated animals fed a high phosphorus diet (NP) equalled the radio-phosphorus levels in those animals on a high phosphorus diet only (P), in 44 out of 48 comparisons. In 47 out of 48 comparisons no difference existed that was statistically significant between the radio-phosphorus content of the teeth of animals treated with norethandrolone (N) and the respective controls (C) at 2, 4, 6, and 8-week intervals.

Radioassay at 2-week intervals was conducted in an attempt to determine if differences existed in the effect of norethandrolone in the more recently erupted teeth compared with those previously formed. The relations of phosphorus uptake in the norethandrolone-treated animals, with or without added phosphorus, to controls were similar at all time intervals. Therefore, a change in the effect of norethandrolone on radio-phosphorus uptake in rat molars compared with controls over a period of time could not be shown. Also, a difference in the effect of the drug on radio-phosphorus uptake between first, second, and third molars compared with controls could not be shown.

It has been stated that norethandrolone had a phosphorus retention effect when administered clinically (14–16). If this is true in the rat, under the experimental conditions in this study, then apparently the increased phosphorus retention had little or no effect upon the uptake of radio-phosphorus in the rat molar. This can be seen from the fact that radiation levels in the norethandrolone animals were equivalent to those in the control group both with and without high phosphorus diets. A further study is in progress to determine if norethandrolone has a phosphorus retention effect in the rat under our experimental conditions.

The most widely accepted opinion of the crystal structure and chemical composition of tooth mineral is that it is basically hydroxyapatite, *i.e.*, $Ca_{10}(PO_4)_6(OH)_2$, although certain deviations, such as substitution of carbonate for phosphate and fluoride for hydroxide, are recognized.

Since phosphorus is an integral part of the hydroxyapatite crystal, it may be assumed that the amount of P^{32} present in molar teeth of rats upon administration of radio-phosphorus is dependent upon three factors. These are (*a*) the production of crystalline hydroxyapatite and its deposition in the matrix, (*b*) ion-transfer across the crystal-solution interface along with intracrystalline exchange, and (*c*) resorption (17).

Little has been done to study the effect of drugs on the physical processes of ion-transfer and intracrystalline exchange in osseous material. It will be necessary at this point, therefore, to assume similarity in this process among all experimental groups within the limits of biological error. There is no reason to assume similarity among experimental groups as far as the metabolic processes are concerned and, therefore, we are relating P^{32} uptake as being indicative of calcification.

Table I shows that the radio-phosphorus content in the norethandrolone-treated rats equalled that found in the respective control groups. This was observed in comparisons of molar teeth from rats fed basic diet plus norethandrolone and Na_2HPO_4 (NP) to rats fed basic diet and Na_2HPO_4 (P), and comparisons of molars from animals fed basic diet and norethandrolone (N) to animals fed basic diet only (C). It would appear that, under the conditions of this experiment, norethandrolone had little or no effect upon calcification.

If norethandrolone had a positive effect upon the synthesis of the collagenous fibrils making up the protein matrix and the mucopolysaccharides making up ground substance, then it could be assumed that a greater rate of calcification would take place. This would seem to be true since Frank (18) showed that hydroxyapatite crystals are laid down on newly formed fibrils of the organic matrix. Also, Belanger (19) found that hydroxyapatite crystal growth occurs soon after the formation of fibrous protein. Carstrom (20) has stated that collagen controls calcification by effecting crystallization of apatite in dentin. In addition, Sobel and his collaborators (21) suggest that, while collagen induces nucleation, the complete system is more complex and probably includes sulfated mucopolysaccharide or mucoprotein. Although it cannot be conclusively stated from this one experiment, it would appear that norethandrolone had little or no effect on collagen synthesis in the rat molar. Further study using radio-sulfur and normal diets and analysis of the radio-sulfur uptake might throw additional light on this particular aspect of the problem. In summary of the above, the results of

TABLE III.—RADIO-PHOSPHORUS LOCATED IN THE MOLAR TEETH OF FEMALE RATS

		Av. c.p.m./mg. ± S.D.					
Animals	Group	Lower First	Lower Second	Lower Third	Upper First	Upper Second	Upper Third
				2-Wk. Interval			
6	1[a]	12.9 ±1.51	12.9 ±1.58	38.4 ±7.00	13.2 ±1.41	12.4 ±1.35	37.3 ±6.99
6	2[b]	13.6 ±1.88	12.9 ±2.08	36.4 ±4.55	14.1 ±1.39	12.7 ±1.81	36.0 ±5.56
6	3[c]	21.5 ±2.68	21.0 ±3.54	65.4 ±7.29	22.4 ±1.12	21.1 ±2.65	57.4 ±9.15
6	4[d]	20.8 ±2.96	20.3 ±2.45	62.3 ±5.07	22.0 ±1.36	20.3 ±2.06	57.5 ±6.71
				4-Wk. Interval			
6	1	13.5 ±1.71	12.6 ±1.93	26.7 ±3.87	12.9 ±1.37	12.2 ±2.02	25.4 ±3.98
6	2	13.7 ±1.58	13.1 ±1.03	23.9 ±1.33	13.5 ±1.48	11.8 ±1.59	24.6 ±1.94
6	3	22.7 ±1.86	22.4 ±2.58	40.8 ±6.89	20.9 ±2.35	20.0 ±1.74	37.4 ±7.69
6	4	21.7 ±2.26	21.1 ±2.40	40.6 ±2.98	20.4 ±1.44	19.2 ±2.21	38.7 ±3.85
				6-Wk. Interval			
6	1	16.0 ±1.15	14.5 ±1.39	24.8 ±1.11	15.8 ±1.50	14.0 ±1.10	24.6 ±2.91
6	2	15.6 ±1.80	14.7 ±1.01	22.6 ±2.24	14.5 ±0.50	13.5 ±1.05	21.3 ±1.45
6	3	24.5 ±1.81	23.3 ±2.21	37.2 ±3.55	25.0 ±2.45	21.5 ±2.16	36.5 ±2.83
6	4	24.8 ±1.67	22.0 ±1.41	35.7 ±3.32	23.1 ±2.21	22.1 ±1.08	34.3 ±4.05
				8-Wk. Interval			
6	1	13.2 ±0.92	11.8 ±1.08	17.0 ±0.53	13.1 ±0.85	12.8 ±1.07	16.8 ±1.40
6	2	12.3 ±1.43	11.8 ±1.63	16.1 ±1.27	13.4 ±2.51	12.0 ±1.46	15.8 ±1.40
6	3	22.8 ±2.90	18.7 ±0.72	28.6 ±4.65	21.5 ±2.50	21.2 ±2.82	28.0 ±2.54
6	4	22.9 ±1.45	21.7 ±3.37	28.1 ±2.45	21.2 ±2.06	21.3 ±1.68	28.5 ±1.77

[a] Group 1 = norethandrolone plus 2% Na_2HPO_4 added to diet. [b] Group 2 = 2% Na_2HPO_4 added to diet. [c] Group 3 = norethandrolone added to diet. [d] Group 4 = basic diet.

this experiment would indicate that the caries resistance of norethandrolone-treated animals fed diets containing 2% disodium phosphate is not related to phosphorus deposition, rate of calcification, or collagen synthesis in rat molars.

A study of Tables II and III shows that the radio-phosphorus content of the teeth in the animals on high phosphorus diets was at a lower value than the radio-phosphorus content in the teeth of animals on low phosphorus diets. This effect was not related to the presence or absence of norethandrolone. This was true in the majority of all comparisons made.

There probably is no one explanation for these results. One reason could be a dilution factor occurring in those animals on a high phosphorus diet. Since the use of a particular phosphate ion from the metabolic pool in synthesis is a random procedure, the greater ratio of nonradioactive ions to radioactive ions would tend to lower the activity in the synthesized dental tissue in those animals on a high phosphorus diet.

Probably of greater importance is the relation of added dietary phosphorus to urinary phosphate elimination. Sivachenko (22) found that heavy administration of nonradioactive phosphorus and calcium salts was most effective in increasing the rate of P^{32} elimination in rats.

Goldman and Bassett (23) studied renal regulation of phosphorus excretion in male patients with normal phosphorus metabolism. They found that daily oral administration of a mixture of sodium and potassium phosphates caused a significant increase in the serum phosphorus level in the patients. This was followed by an increase in urinary phosphorus.

It would appear that the difference in activity in the teeth of high-phosphorus *versus* low-phosphorus animals might be accounted for by these above factors.

CONCLUSIONS

The influence of norethandrolone on radio-phosphorus deposition in rat molars of animals on high and low phosphorus caries-test diets was determined.

Radio-phosphorus levels in the molar teeth of norethandrolone-treated animals fed a high phosphorus diet equalled the radio-phosphorus levels in those animals on a high phosphorus diet alone.

No statistically significant difference existed between the radio-phosphorus content in the molar teeth of animals treated with norethandrolone and the respective control animals.

The radio-phosphorus content of the molar teeth in animals on high phosphorus diets was at a lower value than the radio-phosphorus content in the teeth of animals fed low phosphorus diets. This effect was not related to the presence or absence of norethandrolone but might be accounted for by ion dilution or an increased rate of phosphorus elimination.

No change in the effect of norethandrolone on radio-phosphorus uptake in rat molars, with or without added phosphorus, over a time interval was shown.

A difference in the effect of norethandrolone on radio-phosphorus uptake between first, second, and third molar teeth compared with respective controls was not observed in either animals fed a high phosphorus diet or rats on a low phosphorus diet.

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