the pituitary gland,[7] the dilated state of the melanophores was greater and showed definitely less reflectance than frogs that were dark adapted by environmental stimulation only. Further studies of the same kind in which various dosages of related hormones are given may lead to helpful information concerning the mechanism of chromatophore action in animals.

Summary. (1) The spectrophotometer has been used as an instrument for recording the effects of melanophore expansion on the reflecting power of the skin pigments of the frog. (2) The reflectance in the light adapted frogs may be as much as 10% above that of the dark adapted frog at the same wave length. (3) Melanophore expansion in dark adapted frogs masks the reflecting power of the skin pigment at the red end of the spectrum more than at the violet. (4) The average skin reflectance in the light adapted frogs is not more than 21.8%. (5) A difference between the response of melanophores to environmental conditions and operations of the hypophysis is suggested.

[7] Steggerda, F. R., and Soderwall, A. L., *J. Cell. and Comp. Physiol.*, 1939, **13**, 31.

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Interrelationship of Vitamin D and the Sex Hormones in Calcium and Phosphorus Metabolism of Rats.

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Introduction. The present therapeutic trend for treating chronic arthritis in humans involves the use of high dosages of vitamin D, and has many successes to its credit.[1,2] The favorable treatment of Raynaud's disease in the same manner, in conjunction with sex hormones, has been reported.[3] The high incidence of arthritis in women at a time near to or following the menopause, and the more gradual appearance of this disease in men, is well known. This leads to the suggestion that the role of the sex hormones in these relationships involving the therapeutic use of vitamin D should be more fully understood.

Hypertrophy of the parathyroids has been described in a number of suggestively analogous conditions, viz.: rickets,[4] scleroderma,[5,6,7] Raynaud's disease,[8,9] and chronic arthritis.[10] Experimentally, by perfusion experiments,[11] a low calcium level in the perfusate leads to similar hypertrophy which is overcome by the addition of adequate amounts of calcium. Hyperphosphatemia also induces a corresponding enlargement as one would expect. Vitamin D reduces the compensatory hypertrophy induced by low calcium feedings.[12]

[1] Snyder, R. Garfield, Squires, Willard, Forster, John, Traefer, Cornelius, and Wagner, Lewis Clark, *Indust. Med.*, 1942, **11**, 295.

[2] Wagner, Lewis, *Industr. Med.*, 1942, **11**, 313.

[3] Norman, G. F., *West. J. Surg., Ob. and Gyn.*, 1938, **46**, 553.

[4] Nonidez, J. F., and Goodale, H. D., *Am. J. Anat.*, 1927, **38**, 319.

[5] Cornbleet, T., and Struck, N. C., *Arch. Dermat. and Syph.*, 1937, **35**, 188.

[6] Leriche, R., and Jung, A., *La Presse Med.*, 1938, **46**, 809.

[7] Leriche, R., *Gazette du Hospitaux*, 1936, **109**, 209; Ab. *J. A. M. A.*, 1936, **106**, 1214.

[8] Bernheim, Alice R., *J. A. M. A.*, 1933, **100**, 1001.

[9] Bernheim, Alice, and Garlock, J. H., *Ann. Surgery*, 1935, **101**, 1012.

[10] Wootton, W. T., *J. Missouri M. A.*, 1936, **33**, 129; Abs. *J. A. M. A.*, 1936, **106**, 1854.

[11] Path, Harvey M., Wallerstein, Elizabeth, and Luckhardt, Arno B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 580.

[12] Carnes, William H., Pappenheimer, Alvin M., and Stoerk, Herbert C., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 314.

It also further diminishes the size of these glands already reduced by hypophosphatemia while simultaneously raising both calcium and phosphorus levels in the blood serum.

This apparently occurs independently of the pituitary,[13,14] and the control, if any, of this organ on the parathyroids must occur through the gonads via the gonadotropic hormones.

The effects of overdosage of the parathyroid hormone or the results of tumors of these glands on the organism are well known and need only passing mention here. The demineralization of bone shafts with deformities, the calcification of soft tissues, notably kidneys, lungs, stomach, and arteries, have been described in detail elsewhere.

The present tests were carried out in 1944 and 1945 to investigate the relationship above mentioned, and are now being reported because of the increasing importance of the use of vitamin D in the treatment of arthritis and other conditions. The results obtained were used to support the clinical work undertaken by the senior author.[15]

The present study was undertaken to determine the effect of the addition of estrogens and androgens to rachitic animals administered high doses of vitamin D.

(a) The effect of the addition of the estrogen and androgen were studied in homologous and heterologous fashion.

(b) Criteria of bone-ash were employed in fixing the effect of the addition of vitamin D to a rachitic animal, and also to measure the increment, if any, due to the dosing of the estrogen or androgen, as the case may be.

Experimental. The diet used was Rachitic Diet No. 2, as advocated in the United States Pharmacopoeia Twelfth Revision, supplemented with .1 g each of riboflavin and thiamine hydrochloride per 100 lb of diet. The vitamin D administered orally to the rats was the U. S. P. Reference Oil No. 2. The hormone supplements used were testosterone propionate in sesame oil (Ciba perandren), and estradiol di-propionate in sesame oil (Ciba di-ovocylin). These supplements were injected subcutaneously. Where no hormone supplement was given the rats were injected with Wesson Oil.

All of the animals used in these experiments were of the Long-Evans strain bred from the colony maintained in this laboratory. The young animals had been raised in accordance with U. S. P. standards and were from groups which were available for U. S. P. vitamin D assays. The number of animals assigned to each group varied from 5 to 7 and the groups were adjusted to obtain approximately equal starting weights. The variability within groups due to litter was decreased by the use of isogenic segregation. Litters were chosen which had 3 to 5 animals of the same sex in order that one animal of each litter would appear in each group of a given study; for example: a triad of male rats from the same litter would be found in the control group receiving no vitamin D and no hormone, in the test group receiving only vitamin D, and in the test group receiving vitamin D and the hormone supplement. The rats were kept in individual wire-screen cages and given feed and water *ad libitum*.

In the course of these experiments bi-weekly weights of the animals were taken. At the end of the test period individual blood samples were taken as follows: An incision was made across the belly of the animal, cutting directly into the abdomen, and exposing the vena cava. The vena cava was then severed and the blood sample collected in a centrifuge tube. The chemical analysis of the blood serum was accomplished on a micro scale. Calcium was determined by the permanganate titration method of Tisdall.[16] The inorganic phosphorus was determined by precipitation of the proteins with trichloracetic acid, and a colorimetric determination was made after the reduction of the phosphomolybdic complex with stannous chloride, as advocated by Kuttner and Cohen.[17]

For bone-ash analysis the left tibiae were removed and freed of adhering tissue by buffing with cheesecloth. The bones were

[13] Albright, Fuller, *J. A. M. A.*, 1939, **112**, 2592.

[14] Carnes, Wm. H., Osebold, John, and Stoerk, Herbert C., *Am. J. Physiol.*, 1943, **139**, 188.

[15] Norman, G. F., *Geriatrics*, 1947, **2**, 24.

[16] Tisdall, F. F., *J. Biol. Chem.*, 1923, **56**, 439.

[17] Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, **75**, 517.

TABLE I.
Experiment I.
Effect of the Injection of Hormones in Vitamin D Depleted Rats Fed Various Doses of Vitamin D.

Group	Vitamin D: Daily dose, mg	Vitamin D: Total fed, mg	Hormone dosage: Medication	Hormone dosage: Total dose, mg	Net gain: Mean, g	Net gain: Std. Dev.	Tibia bone-ash %
			Males (Paired Litter Mates).				
1	0	0	Wesson Oil	0	139	± 3.87	31.12
2	6.0	210.0	" "	0	180	± 2.50	48.47
3	6.0	210.0	Perandren	17.5	148	± 4.03	46.22
7	.9	31.5	Wesson Oil	0	170	± 5.62	35.19
8	.9	31.5	Di-ovocylin	17.5	104	± 3.60	42.43
			Females (Paired Litter Mates).				
4	0	0	Wesson Oil	0	179	±3.03	36.36
5	6.0	210.0	" "	0	203	± 5.03	50.69
6	6.0	210.0	Di-ovocylin	3.5	88	± 3.35	53.53
9	.9	31.5	Wesson Oil	0	235	± 5.74	44.66
10	.9	31.5	Perandren	17.5	212	± 4.51	38.16

extracted in anhydrous isopropanol for 2 hours and then ashed by group.

On the next to the last day of the test the animals were anesthetized and individual X-rays taken of each rat. Representative X-rays are presented to show the skeletal development which resulted from the studies undertaken.

In the preliminary studies young male rats were depleted for 18-21 days on the rachitic diet and then placed on assay for 10 days under conditions prescribed in the United States Pharmacopoeia. The daily dose of vitamin D was fed orally, and hormone supplements administered subcutaneously at the start of the assay and on the fifth day of assay. Results obtained indicated that although a large difference in percent bone-ash was obtained between the control group fed no vitamin D (29.50) and the highest level of vitamin D fed 10.67 mg per day (34.61), the 10-day test period would not allow for significant changes due to the injection of the hormone. It was felt that a longer assay period would allow for more treatments and, hence, a more critical study of the role of the hormone supplements. Therefore, in the assays herein reported the "prophylactic bone-ash method" as advocated by Coward[18] was used. When the rats were weaned they were placed directly on the rachitic diet and the assay started, continuing for 5 weeks. The dose of vitamin D was one-fifth of the curative test and given twice weekly instead of daily. The hormone supplement was injected every 5 days (Sundays excluded).

Results and Discussion. As advocated by Coward,[18] the level of vitamin D to be fed in the "prophylactic bone-ash method" was one-fifth of the dose use in the curative U. S. P. assay required to give a 2-plus healing. The level of vitamin D used on assay designated as the high dose was 5 times the level required to give a 2-plus healing.

Sixty rats, ranging in age from 21-24 days, and in weight from 40-63 g, and consisting of 30 males and 30 females, were allotted into 10 groups, as shown in Table I. The 5 male groups were made up of litter-mate-brothers, and similarly the 5 female groups of litter-mate-sisters. One of the 5 groups served as the control for the 2 test groups involving the treatment with the homologous hormone, and also as the control for the other 2 groups devoted to the treatment with the heterologous hormone. The mean net gain per group, its standard error, and the percent bone-ash of the extracted tibiae are summarized in Table I.

In order to determine statistically whether or not the mean difference observed in Table I between Groups 5 and 6 was significantly different, the right tibia of each animal was prepared as in the case of the group ashings

[18] Coward, K. H., *Biological Standardisation of the Vitamins*, Baillière, Tindall and Cox, London, 1938, 121.

TABLE II.
Experiment I.
Statistical Analysis of Data.

Group 5: Females; vitamin D—high dose.
Group 6: Females; vitamin D—high dose plus Estrogen.

	Tibia Bone-ash %	
	Group 6	Group 5
	56.72	43.73
	51.83	49.09
	53.85	46.94
	53.79	49.09
	51.21	51.94
	54.04	54.43
Mean	53.57	49.20
Mean diff.		4.37
Stand. dev. of diffs.		5.14
Stand. error of mean diffs.		2.099
Critical ratio (t)		2.08

With 5 degrees of freedom the value for t when P equals .10 is 2.015, and the value found exceeds this limit.

except that now the bones were individually labeled, extracted, and ashed.

Since these groups were made up of 6 pairs of sisters the results obtained were treated according to "Student's" method of analysis for correlated data as shown in Table II.[19]

From Table I it will be seen that females tolerate depletion better than males, as indicated by the higher percent bone-ash for the control group (Group 4 vs. Group 1). This could possibly be explained by the presence of ovarian tissue.

It is obvious that the addition of a large amount of vitamin D led to an increase in body weight in both sexes, while the added medication counteracted the increase due to vitamin D.

In males the employment of estrogen significantly increased the percentage of bone-ash (Group 7 vs Group 8), while this was diminished with androgen as the medication (Group 2 vs. Group 3).

The use of the high dosage of vitamin D in the females definitely enhanced the percent bone-ash, and this was further increased with the use of the estrogen (Group 5 vs. Group 6). On the low dose of vitamin D the females were particularly sensitive to the androgen, definitely decreasing the percent bone-ash (Group 9 vs. Group 10). In this instance 2 factors apparently operate, namely, the low protection of the vitamin D dosage and the parathyroid stimulating effect of the androgen.

As shown in the statistical analysis in Table II, a significant increase in the percent bone-ash was observed when female rats given a high dose of vitamin D were concurrently treated with estrogen (Group 5 vs. Group 6).

In the first experiment with female rats the use of the estrogen definitely enhanced the percent bone-ash when given in connection with the high dose of vitamin D, and similarly showed an increase for males when the low dose of vitamin D was administered. On the other hand, the use of the androgen decreased the percent bone-ash when given in connection with the low dose of vitamin D to females, and also showed the same effect when given with the high dose of vitamin D to males.

These findings are in accord with the observations of Gardner,[20,21] who investigated the effect of estrogens and androgens on the breaking strength of femurs of mice. However, the use of rachitic animals and the employment of sex hormones in conjunction with vitamin D have not been considered. In addition, the increase in bone-ash attributed to the use of the estrogen confirms the work of Day and Follis.[22] It was proposed, therefore, to continue the study of the use of the androgen in connection with the high dose of vitamin D administered to both males and females.

Thirty-seven rats, ranging in age from 23-26 days, and in weight from 47-74 g, and consisting of 22 males and 15 females, were allotted into 6 groups, as shown in Table III. The 3 male groups were made up of litter-mate-brothers, and similarly the 3 female groups of litter-mate sisters. One of the 3 groups served as the control for the 2 test groups involving the treatment with perandren.

The 3 male groups consisted of 8 animals

[19] "Student," *Metron.*, 1925, **5**, No. 3, 114.

[20] Gardner, W. U., *Endocrinology*, 1943, **32**, 149.

[21] Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 230.

[22] Day, Harry G., and Follis, Richard H., Jr., *Endocrinology*, 1941, **28**, 83.

TABLE III.
Experiment II.
Effect of the Injection of Androgen in Vitamin D Depleted Rats Fed Various Doses of Vitamin D.

Group	Vitamin D: Daily dose, mg	Vitamin D: Total fed, mg	Androgen dosage: Medication	Androgen dosage: Total dose, mg	Net gain: Mean, g	Net gain: Std. Dev.	Tibia bone-ash, %	Analysis of serum: Calcium, Mean, mg %	Analysis of serum: Inorganic phosphorus, Mean, mg %
				Males (Paired Litter Mates).					
1	0	0	Wesson Oil	0	37.00	± 4.18	34.44	9.98	3.7
2	6	210	" "	0	37.43	± 4.70	49.03	10.57	3.6
3	6	210	Perandren	17.5	34.86	± 3.92	47.29	9.63	3.8
				Females (Paired Litter Mates).					
4	0	0	Wesson Oil	0	33.20	± 1.48	35.57	9.20	3.8
5	6	210	" "	0	29.00	± 3.85	50.60	10.80	3.1
6	6	210	Perandren	17.5	28.20	± 3.25	49.09	9.54	3.9*

* Mean of 3 samples.

in the control and seven in each of the test groups, while the 3 female groups were made up of 5 animals each. The mean net gain per group, its standard error, and the percent bone-ash of the extracted tibiae are summarized in Table III.

In the first experiment blood samples were taken, but were only used to establish the technic for handling the samples taken in this experiment. Therefore, in addition, Table III contains a summary of the analysis of the serum with respect to the calcium and inorganic phosphorus.

From Table III it will be observed that with both sexes there was a gain in weight in the animals employing vitamin D which was equal to the controls, as well as a definite increase in the percent bone-ash of the tibia. The use of androgen again diminished the percentage of bone-ash, and also reduced the net gain in weight when employed in conjunction with the vitamin D.

In the blood serum there were apparently no significant changes in the inorganic phosphorus level; however the calcium levels were depressed with the use of the androgen as the medication.

In order to determine statistically whether or not the mean difference observed in Table III between Groups 2 and 3 was significantly different, the right tibia of each animal was prepared as in the case of the group ashings except that now the bones were individually labeled, extracted, and ashed.

Since these groups were made up of 7 pairs of brothers the results obtained were treated according to "Student's" method for analysis for correlated data as shown in Table IV.[19]

TABLE IV.
Experiment II.
Statistical Analysis of Data.

Group 2: Males; vitamin D—high dose.
Group 3: Males; vitamin D—high dose plus Androgen.

	Tibia Bone-ash %	
	Group 2	Group 3
	50.35	49.29
	45.78	46.39
	48.39	46.12
	50.84	51.26
	46.50	46.63
	50.78	47.83
	47.69	44.69
Mean	48.62	47.46
Mean diff.		1.16
Stand. dev. of diff.		1.588
Stand. error of mean diff.		.600
Critical ratio (t)		1.933

With 6 degrees of freedom the value for t when P equals .10 is 1.943, and the value found approximates this limit.

The individual X-rays of the rats employed in the 2 tests were critically examined and the changes in bone structure noted. With respect to both sexes, the female sex hormone increased the density of the bone-shaft and indicated an advanced epiphyseal closure. The male sex hormone showed an opposite effect on both sexes as evidenced by a diminution of calcification of the bone-shaft and a slowed epiphyseal closure. These changes are best illustrated in representative photographs

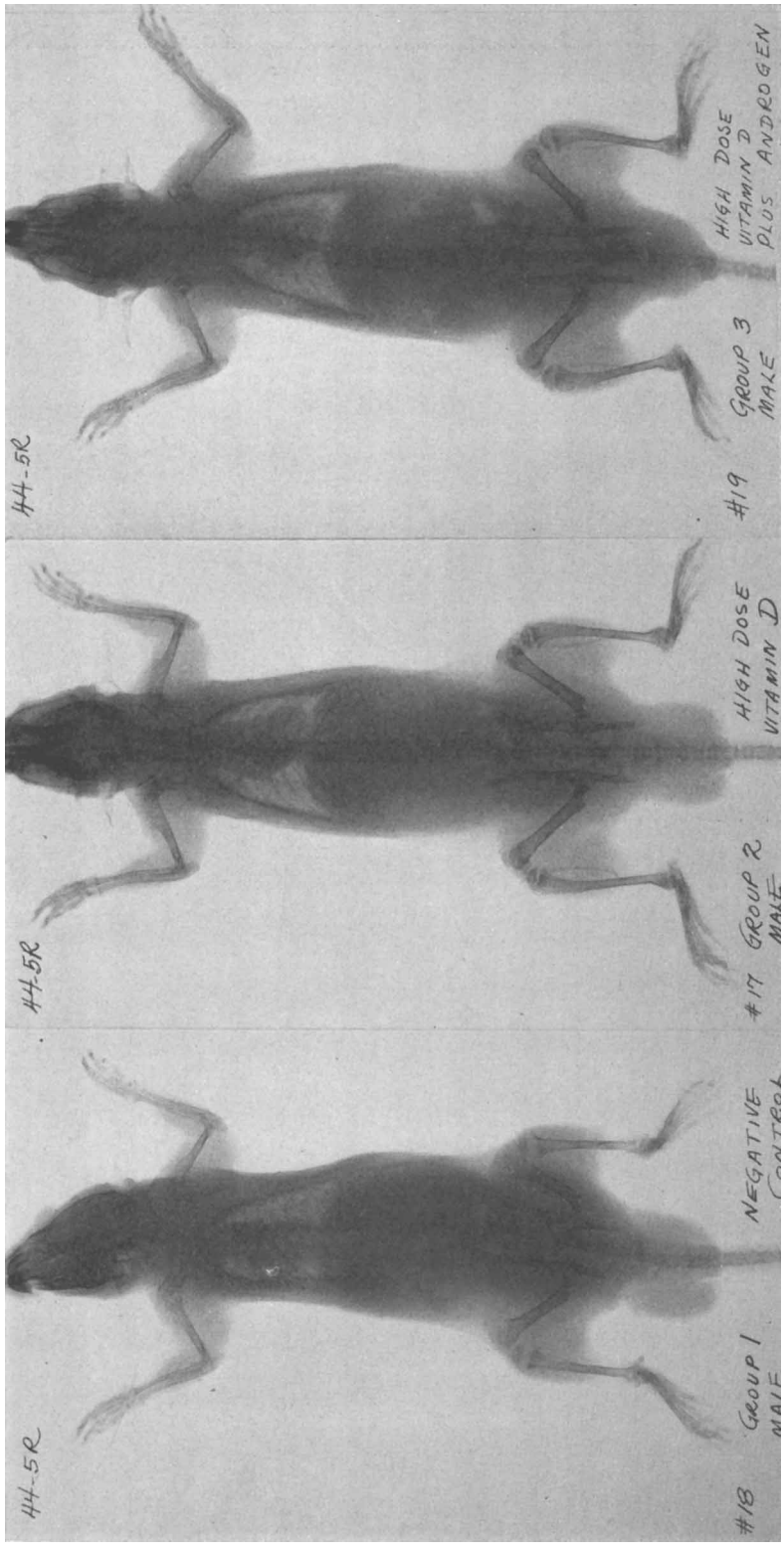
44-5R
#18 GROUP 1
MALE
NEGATIVE
CONTROL
44-5R
#17 GROUP 2
MALE
HIGH DOSE
VITAMIN D
44-5R
#19 GROUP 3
MALE
HIGH DOSE
VITAMIN D
PLUS ANDROGEN

FIG. 1.

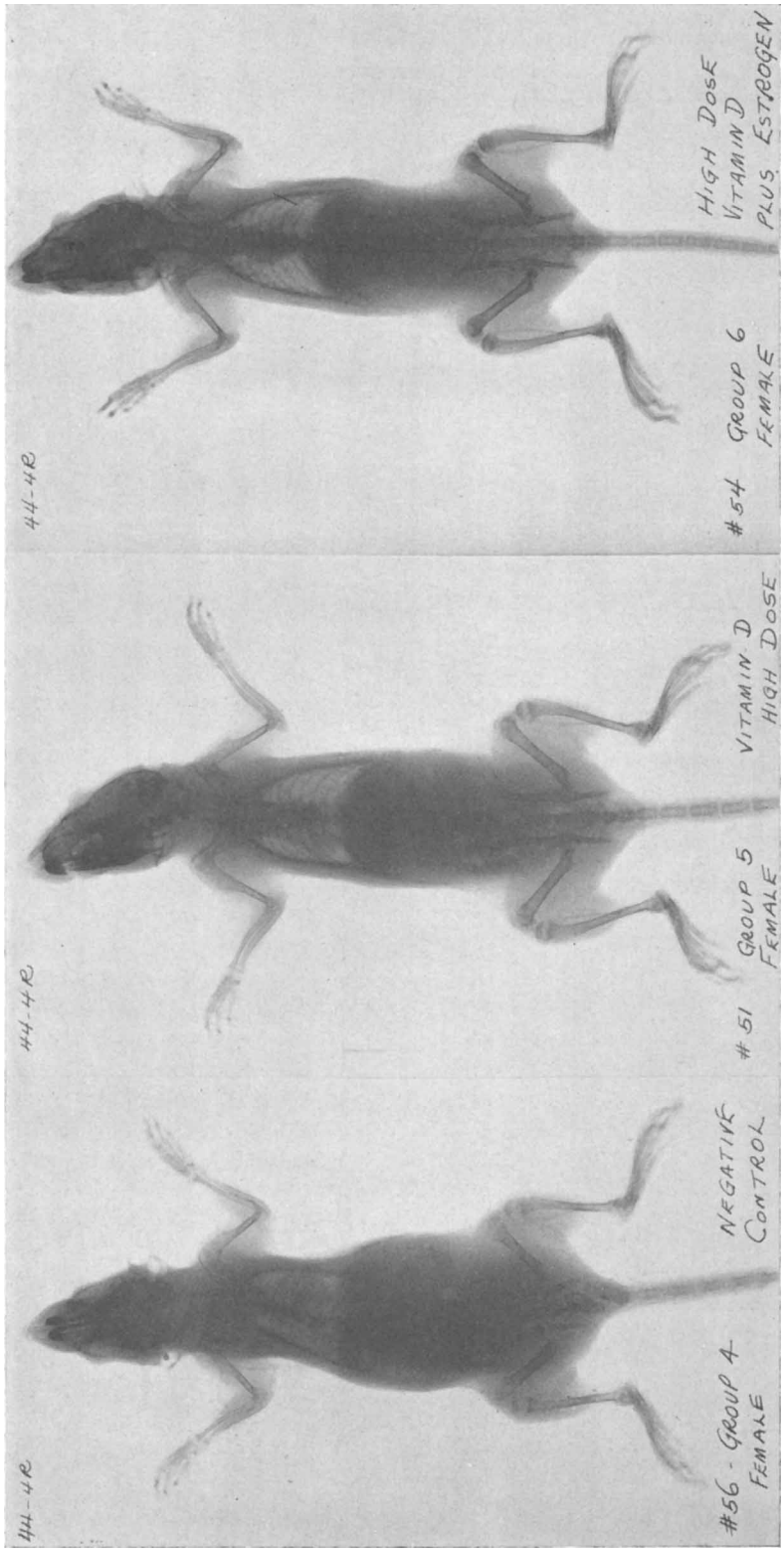
44-4R
#56 - GROUP 4
FEMALE
NEGATIVE
CONTROL
44-4R
#51
GROUP 5
FEMALE
VITAMIN D
HIGH DOSE
44-4R
#54
GROUP 6
FEMALE
HIGH DOSE
VITAMIN D
PLUS ESTROGEN

FIG. 2.

of sets of litter mates taken from the assay procedures.

Conclusion. On the basis of these experiments carried out with rats, it is our conclusion, in agreement with other observers, that for animals of both sexes the female sex hormone (a) stunts the growth of young animals, (b) increases the density of the bone-shaft, (c) accelerates epiphyseal closure, (d) increases the percentage of bone-ash.

These postulates confirm the depressing effect of this hormone on the parathyroid glands; further confirmed by the greater response in this effect when female animals were used (Experiment I).

The male hormone, on the other hand, in both sexes (a) stunts the growth of the animal, (b) diminishes the density of the bone-shaft, (c) diminishes the percentage of bone-ash, (d) delays epiphyseal closure, (e) depresses slightly the blood calcium, but does not increase the serum inorganic phosphorus.

To summarize: The male sex hormone is rachitogenic; the female sex hormone is antirachitic. The use of the female hormone is suggested either alone or in conjunction with vitamin D in the treatment of either sex where there is overaction of the parathyroids.

The authors wish to thank the California Packing Corporation and Mr. J. E. McConkie, Director of Research of that organization, for their splendid cooperation in these vitamin studies, and to the Ciba Pharmaceutical Company for the liberal contribution of their endocrine products used in these studies.

16219 P

Activation of Plasma Thromboplastinogen and Evidence of an Inhibitor.*

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Evidence has been presented earlier indicating that the first reaction in the coagulation of the blood is the conversion of the precursor of thromboplastin, thromboplastinogen, to the active form.[1] This is brought about by a factor in the platelets. Hemophilic platelets appear equally as active as those of normal blood. The reaction can readily be studied by the prothrombin consumption test, which consists in determining the prothrombin before and after coagulation. Typical results are presented in Table I.

Recently Milstone[2] has also obtained data showing that the thromboplastin in plasma occurs in an inactive form which he names prothrombokinase. He observed that it is activated in the presence of calcium, but he does not mention or apparently consider the platelets as a possible activation factor. From recent studies[1] it appears more likely that thromboplastin is not an enzyme but that its activation is enzymatic.

Recently it was found that the blood of a patient who developed a hemophilia-like disease (presumably as a sequela to pemphigus) contains an agent which inhibits the activation of thromboplastinogen. Significant results obtained in the study of the patient's blood are given in Table II. In addition it was found that the prothrombin time response of the patient's plasma to serial dilutions of thromboplastin is identical with that of normal plasma.

It seems clear that the impaired coagulation is not due to an anti-thromboplastin. The cause must be due either to a lack of thromboplastinogen or to failure of the latter

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1 Quick, A. J., *Am. J. Med. Sci.*, 1947, **214**, 272.

2 Milstone, J. H., *Science*, 1947, **106**, 546.