Summary: Fibrinolytic determinations made with 33 strains of hemolytic streptococci isolated from different conditions showed individual variations in their lytic activity when tested under similar conditions. Apparently, the plasma clot derived from cases of recurrent tropical lymphangitis develops a definite resistance to the fibrinolytic activity of hemolytic streptococci.

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Refinements in X-ray Technique for the Estimation of Vitamin D.*

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None of the existing techniques commonly employed for the estimation of Vitamin D are entirely beyond criticism. In choosing the most suitable procedure a number of factors must be considered, depending upon whether time and economy or accuracy and reliability are to receive the most consideration. To indicate briefly some of these factors, we have but to point out the principal advantages and limitations of the 3 widely used methods for the assay of antirachitic potency, in all of which inbred stocks of albino rats furnish the experimental animals.

Since all 3 methods may be adapted to either curative or preventive procedures and since the former enjoy by far the greater vogue in this country, the present discussion and experimental work are limited to curative methods. The Steenbock diet No. 2965 was used to produce rickets, and, in general, the recommendations of the Committee of the American Drug Manufacturers Association on Vitamin Assay were followed.[3]

The bone-ash technique as worked out principally by Chick, Roscoe and others[1, 2] is a purely objective procedure, not subject to aberrations of human judgment, even to the extent that they occur in the other 2 methods. On the other hand, the factor of biological variation exerts its greatest influence in this method, and the statis-

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[1] Chick and Roscoe, *Biochem. J.*, 1926, **20**, 137.

[2] Chick, Korenchevsky, and Roscoe, *Biochem. J.*, 1926, **20**, 622.

[3] Holmes, *Rep. A. D. M. A., Com. Vit. Assay*, 1932.

tical significance of the results can be increased only by proportionally lengthening the series of experimental observations in order that the average figures may not be unduly influenced by such factors as variation in animal stature, susceptibility to rickets, etc.

The "line-test"[7] furnishes the most convenient method from the standpoint of time and equipment and can be brought to a high degree of dependability.[3] Here also, however, animal variation may markedly influence the results, particularly variations in susceptibility to the effects of the rachitogenic diet. The only check possible on this point is that obtained by preliminary tests done on random animals before the beginning of the experimental period. Any adequate line-test procedure includes photographing the specimens as soon as the "line" has been developed in order that permanent records may be obtained.[4] A convenient camera for this work was constructed. For the tibia, the negative is made at unit magnification on fine grain film. Prints may be made at magnifications up to 10 diameters without film grain becoming noticeable.

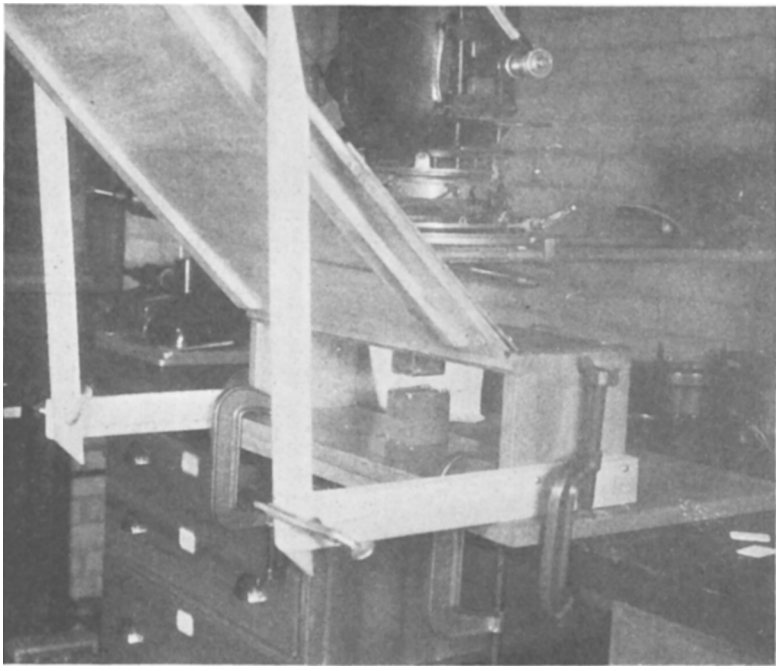


FIG. 1.
Rat and film holder with lead shields.

[7] McCollum, Simmonds, Shipley and Park, *J. Biol. Chem.*, 1922, **51**, 41.
[4] Stevens and Nelson, *Ind. Eng. Chem. Anal. ed.*, 1932, **4**, 200.

One of the first X-ray techniques at all comparable to the line-test was suggested by Poulsson and Lovenskiold.[5] The idea has been further elaborated by Bourdillon, et al.[6] Since X-ray photographs of each individual animal are taken before the beginning of the experimental period, anomalous cases can be recognized and the chief objection to the other 2 methods is overcome, in that variations in susceptibility to the rickets-producing diet do not weight the final results. One serious objection to these techniques, however, lies in the fact that only 2 X-rays are taken, one at the beginning of the experimental period, under anesthesia, and the other at the end of the test after the animals have been killed. While more frequent radiography is desirable, increasing the amount of anesthesia is to be avoided as it may frequently lead to increased incidence of respiratory infection and other metabolic upsets among the experimental animals.

To overcome this difficulty, and to avoid the use of strenuous methods of clamping the rats in position for photographing, which often results in injury, the present modification was developed. Duplitized dental film was held in a special holder (Fig. 1) of such dimensions, and so shielded with sheet lead as to allow both fore and hind legs of the rats to be held in place for exposure with-

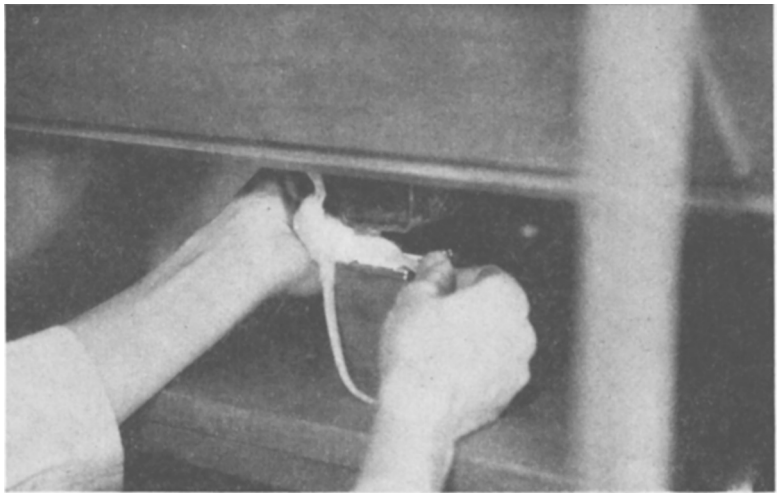
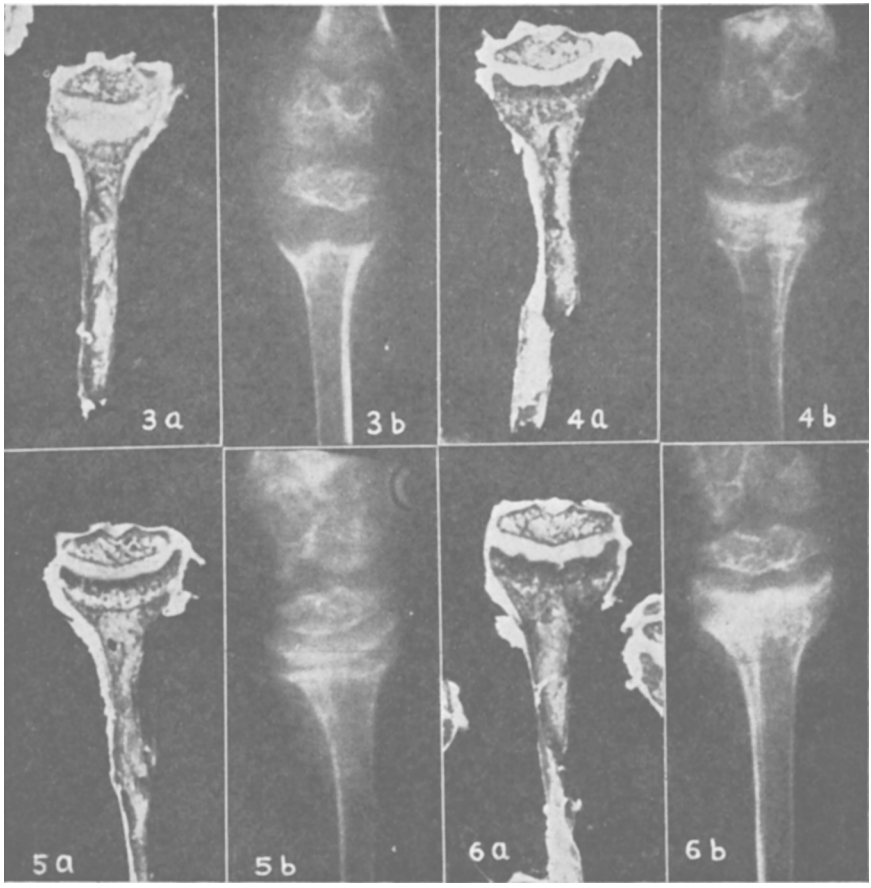


FIG. 2.
Method of holding rat for X-ray photograph of tibial head.

[5] Poulsson and Lovenskiold, *Biochem. J.*, 1928, **22**, 135.

[6] Bourdillon, Bruce, Fischmann and Webster, Med. Res. Council Special Report Series No. 158.



FIGS. 3-6.

3 a, b. Line-test and X-ray of rachitic rat.

4 a, b. Line-test and X-ray of healed rickets. Total dose, 16 international units in form of irradiated milk.

5 a, b. Line-test and X-ray showing effect of total dose of 9 units of international standard solution of vitamin D.

6 a, b. Line-test and X-ray showing effect of total dose of 10 international units in form of irradiated milk.

out endangering the hands of the operator. (Fig. 2.) The dental packets were placed in a slide and pushed against a stop. Suitable clips were provided to hold lead stencil numbers for identification. With a little practice, the rats may be held quite easily and loosely without showing any of the fine muscular tremor which accompanies mechanical clamping. There was very little spoilage of film.

We feel that the advantages accruing from the X-ray estimation of Vitamin D by the present technique are obvious. Each animal is checked before the beginning of the test feeding period to estab-

lish the degree of rickets. The progress of healing is followed in each animal at frequent intervals so that any anomalies are recognized. Permanent records are provided and may be re-read at any time. While subjective, the estimations, if based on a comparison of the unknown antirachitic substance with a standard, provide a completely null method of observation. Thus, different observers comparing the X-rays may be expected to arrive at similar results. The averages compare closely with either line-test or bone-ash averages on the same animals, and the spread of observations is certainly no greater than that found with the other methods.

A comparison was made between line-test, X-ray and bone ash estimations on a small series of animals (18 in all) used to determine the potency of an irradiated milk in terms of the International Standard Vitamin D solution. The results (Table I) represent independent estimations by the 3 methods uninfluenced by the other 2. It is felt that the agreement is very good considering the few animals used.

TABLE I.

Feeding Level International Units per day	International Units per cc. of Milk		
	Bone Ash	Line Test	X-Ray
0.5	0.50	0.40	0.40
1.0	0.45	0.40	0.40
2.0	0.50	0.53	0.50
Average	0.48	0.44	0.43

We are indebted to Dr. E. M. Luce-Clausen, who very kindly made the bone ash determinations reported here.

It seems to have been generally recognized among workers in the field, although not, to our knowledge, specifically pointed out in the literature, that the nature of the calcification is by no means independent of the type of antirachitic substance fed. In particular, when an irradiated milk is compared with an irradiated ergosterol concentrate, it has been noticed that the initial calcification is frequently more diffuse in character, "a narrow and continuous line of calcification" seldom occurring in the milk-fed animals.

Examples of the line-test and X-ray photographs on the same animals are shown in Figs. 3 to 6 at equal magnification. Not only the degree but also the character of the healing is shown. In Fig. 5, the narrow line of calcification (animal fed International Standard irradiated ergosterol solution) is shown in marked contrast to the diffuse calcification occurring in an animal fed irradiated milk at substantially the same potency level, Fig. 6.