diation. This concept is presently being further investigated for it may demonstrate that it is possible to have a local aging of tissues as contrasted to the generalized effects. This accumulation could be caused by an increased synthesis, or by a decrease in turnover rate caused by the ionizing radiation. In either circumstance the net result would be an increase in fibrous material.

Summary. Radioactive capsules containing 1 mC of Co^{60} when imbedded in subcutaneous induced granulomas, significantly decreased the amount of collagen present. This decrease is due mainly to a change in the rate of synthesis, as judged by the considerable drop in the amount of soluble collagen precursors present in the irradiated granulomas. Hexosamine concentration in developing granuloma was not significantly affected by irradiation. The necrotic skin above the granulation tissue in the irradiated animals showed an increase in total collagen, presumably of a relative nature due to a depletion of soluble tissue constituents. The rate of synthesis at this site was decreased as judged by a drop in soluble collagen precursors extractable by isotonic NaCl solution.

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1. Nimni, M., Gerth, N., Bavetta, L., Rad. Res., 1965, v26, 221.
2. Fletcher, G. H., Wootton, P., Storey, W. H., Shalek, R. J., Am. J. Roentgenol., 1954, v71, 1021.
3. Nimni, M., Bavetta, L., Proc. Soc. Exp. Biol. and Med., 1964, v117, 618.
4. Woessner, J. F., Jr., Arch. Biochem. Biophys., 1961, v93, 440.
5. Rondle, C. J. M., Morgan, W. T. J., Biochem. J., 1955, v61, 586.

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Measurement of Non-Crystalline Calcium Phosphate in Bone Mineral.* (31073)

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Bone is a connective tissue with extracellular substance consisting of a calcium phosphate mineral dispersed in what is essentially a hydrated collagen matrix. It is well known that the X-ray diffraction pattern of bone mineral is characteristic of an apatite structure. On the basis of this evidence and chemical analysis, it is thought that bone mineral consists of a crystalline phase similar to, but not identical with, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). The purpose of this paper is to show that the periodic structure giving rise to the X-ray diffraction peaks does not account for all of the mineral present in bone and that, in fact, there must be at least a single major calcium phosphate phase present which is non-periodic, or amorphous, in nature. This investigation has been stimulated by the recent finding that a non-crystalline calcium phosphate is the precursor phase in the preparation of hydroxyapatite under basic conditions(1,2).

Materials and methods. All materials studied were ground to 300 mesh, air dried and examined by standard X-ray diffraction techniques in a *Siemens* scintillation counter, recording diffractometer. The operating conditions were: 37 Kv, 38 ma, Copper target, nickel filter, percentage counting error 0.5%, $1/8°2\theta$ per minute diffractometer scan speed, 4 mm exit slit, 1.2 mm entrance slit for lower angle region and 2.4 mm slit for higher angle region. The X-ray powder data for each sample were obtained in 2 regions, peak No. 1 between $27.25°2\theta$, and $24.50°2\theta$ and Peak No. 2 between $41.25°2\theta$ and $37.50°2\theta$ (Fig. 1).

The moisture content of each sample was determined by the difference in weight after air drying and drying for 18 hours at 110°C. The ash weight was determined by the difference in weight between the air dried sample and the sample heated for 18 hours at 600°C.

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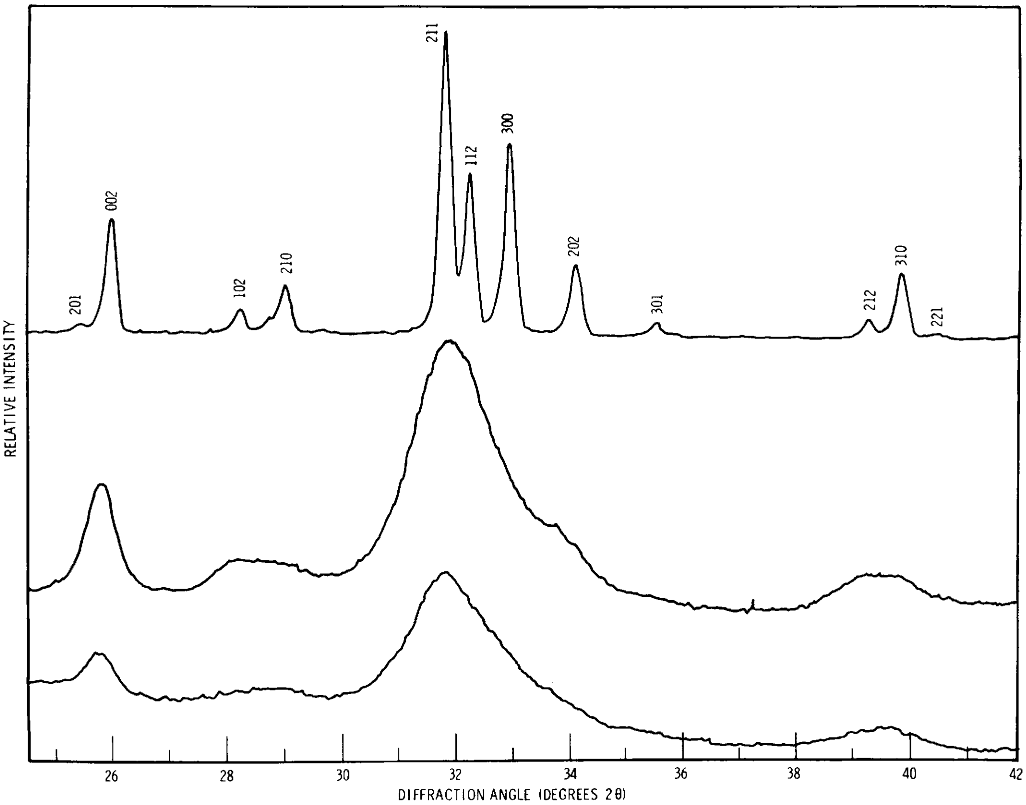

FIG. 1. X-ray diffraction patterns (Copper $K\alpha$ radiation) of powdered: (a) bone femur diaphysis (lower pattern), (b) standard synthetic hydroxyapatite (middle pattern), and (c) well crystallized hydoxyapatite (upper pattern). The latter shows the indexed maxima involved in Peak 1 (27.25 - 24.50°2θ) and Peak 2 (41.25 - 37.50°2θ) used in analysis of the noncrystalline fraction of bone mineral. The 2 lower patterns are reproduced to the same scale while the upper pattern is shown at a convenient, arbitrary scale.

The X-ray diffraction measurement should be performed on the day that the air-dry weight was obtained.

Orientation problem. In the X-ray diffraction pattern of a completely random bone powder sample the area under any diffraction peak or any linear combination of peak areas above background would be directly proportional to the amount of crystalline apatite present. Peak No. 1 is related to the 002 reflection while Peak No. 2 is in general related to hkO reflections. Since a non-random orientation of the crystals in the diffractometer holder would result in a change in area under a given peak relative to certain other peaks, such orientation must be eliminated or taken into account. The standard method of randomizing a sample for X-ray studies is to dilute the material with low density, irregularly shaped particles such as powdered cellulose acetate. To avoid modifying the bone samples an empirical method was developed to compensate for orientation effects.

It was found empirically that the sum of the area under Peak 1 and half the area under Peak 2 was constant for a given bone sample regardless of the orientation. This is illustrated in Table I which lists the X-ray results on powdered, mature cow, rat and human femurs when each sample is packed in the diffractometer holder in different ways resulting in different sample orientations.

The 3 preparations for each bone specimen were as follows: (1) The sample was packed normally into the X-ray diffraction powder holder taking no care to avoid orientation; (2) the sample was packed as loosely as possible to avoid orientation; (3) the sample

TABLE I

Preparation	Peak 1 Relative peak area	Peak 2 Relative peak area	Peak 1 + ½ Peak 2 Relative peak area
Human — 1	787	1946	1760
" — 2	897	1836	1815
" — 3	1065	1385	1757
Rat — 1	1104	2094	2151
" — 2	1108	2056	2136
" — 3	1223	1843	2144
Cow — 1	625	2734	1992
" — 2	885	2196	1983
" — 3	1125	1740	1995

was randomized by mixing half and half with cellulose acetate powder and the resulting specimen was packed loosely into the holder. The relative area values for preparation 3 were corrected for the dilution of the sample by cellulose acetate.

X-ray analysis. The integrated intensity (Relative Peak Area) of Peak No. 1 plus one-half of Peak No. 2, shown to be independent of sample packing, was used to determine whether bone mineral contained more than just an apatite phase. It should be noted that the method that follows is in general an adaptation of the technique of quantitative phase analysis described by Alexander and Klug(3).

Since one bone may differ from another in ash and water content the relative peak areas much be corrected as follows:

$$I_m = I_b \frac{W_m(\bar{\mu}_m - \bar{\mu}_n) + \bar{\mu}_n}{W_m \bar{\mu}_m}$$

I_m = relative area for pure bone mineral.
I_b = relative area for air-dried, whole bone (Peak 1 + ½ Peak 2).
W_m = weight fraction of bone mineral in whole bone (*i.e.*, ash weight).
$\bar{\mu}_m$ = average mass absorption coefficient of bone mineral.
$\bar{\mu}_n$ = average mass absorption coefficient of the non-mineral constituents (water, collagen, etc.).

The value of $\bar{\mu}_n$ is particularly sensitive to both the moisture content (W_{H_2O}) and the organic fraction (W_c) of bone, since the mass absorption coefficients of water and the organic fraction are 10.26 and 7.98, respectively. The latter value was calculated from a chemical analysis of the organic matrix of human bone reported by Woodward(4). Thus

$$\bar{\mu}_n = 10.26\, W_{H_2O} + 7.98\, W_c.$$

For purposes of calculating $\bar{\mu}_m$, it was assumed that bone mineral contained phosphorus and oxygen in the same molar ratio as in the mineral hydroxyapatite. On the other hand, the value of $\bar{\mu}_m$ was corrected for a change in Ca/P ratio. The calculation was done in the following manner:

$$\bar{\mu}_m = \frac{[(Ca/P)/1.67]\,10\,\mu_{Ca} A_{Ca} + 6\,\mu_P A_P + 26\,\mu_O A_O}{[(Ca/P)/1.67]\,10\,A_{Ca} + 6\,A_P + 26\,A_O}$$

where,

Ca/P = molar ratio of Ca to P,
μ = mass absorption coefficient of atom designated by subscript, and
A = atomic weight of atom designated by subscript.

Note that when the Ca/P is 1.67, the value for hydroxyapatite, the $\bar{\mu}_m$ value is that of stoichiometric hydroxyapatite. Since the value of $\bar{\mu}_m$ is relatively unaffected by such atomic bone constituents as carbon and hydrogen the effect of these elements was not considered in the calculation of $\bar{\mu}_m$.

A standard sample of hydroxyapatite, prepared as indicated earlier(1), "fully converted" to the crystalline state, was selected so that it yielded an X-ray diffraction pattern identical in shape with that of the apatite portion of the patterns obtained from the bone samples studied. (The bone apatite patterns of all specimens in this study were appreciably the same.) Thus, the standard hydroxyapatite had the same crystal size and/or crystal perfection as the bone apatite samples but it contained no other phase but a crystalline hydroxyapatite. In the selection of a standard the important factor was to use a fully crystalline hydroxyapatite yielding an X-ray pattern which matched the bone patterns in shape. It was found empirically that the exact stoichiometry of the standard was unimportant as long as all of the amorphous precursor, which occurs in the precipitation (1) was fully converted to the crystalline state.

It is possible to estimate the fraction of apatite present in bone by comparing the relative area, I_{HA}, of the fully crystalline standard with the relative area, I_m, obtained from bone. The I_m value of the bone apatite, corrected for water, organic matrix and Ca/P ratio, as above, is related only to the weight fraction of apatite present. If the bone mineral is composed of nothing but crystalline material, I_m should be identical to I_{HA}. The "mismatch" of these two values is the key to the non-crystalline calcium phosphate fraction of bone mineral. Thus:

$$(I_m)/(I_{HA}) = 1 - W_{am},$$

where W_{am} is the weight fraction of calcium phosphate which does not contribute to the crystalline X-ray diffraction pattern and thus is in a non-crystalline state. The per cent non-crystalline is obtained, of course, by multiplying the value of W_{am} by 100.

A short discussion of the derivation of this relationship may be in order. One can treat bone mineral as a binary mixture of crystalline and amorphous phases. Thus, the ratio I_m/I_{HA} can be represented as follows (3):

$$(I_m)/(I_{HA}) = (W_{HA}\,\mu_{HA})/[W_{HA}(\mu_{HA} - \mu_{am}) + \mu_{am}]$$

where W_{HA} = weight fraction of crystalline hydroxyapatite,
μ_{HA} = mass absorption coefficient of hydroxyapatite, and
μ_{am} = mass absorption coefficient of the amorphous calcium phosphate.

If we assume $\mu_{am} = \mu_{HA}$, the ratio, as stated above, becomes:

$$(I_m)/(I_{HA}) = W_{HA} = (1 - W_{am}).$$

X-ray diffraction studies were made on the following sample series in order to check the validity of the method and to apply it to the case of bone.

1) Ten samples, made up of varying proportions of (a) the standard 100% crystalline hydroxyapatite and (b) synthetic amorphous calcium phosphate (Ca/P = 1.48) (prepared as described in reference 1), were examined and the X-ray diffraction relative areas (I_m) were plotted against percent of non-crystalline calcium phosphate (Ca/P = 1.64) in the samples.

2) Five of the samples in 1 were admixed, 50% by weight, with bone matrix obtained by the EDTA demineralization of bone. This series required correction for the presence of water and organic matrix to obtain the relative areas.

3) A series of bone samples prepared in different ways were analyzed for the percent of the non-crystalline phase present in the mineral. In addition to studying untreated cow and Wistar rat femurs, analyses were made on Wistar rat femurs after (1) alcohol-ether extraction (4 hours each), (2) refluxing in anhydrous ethylene diamine for 4 hours, and (3) both alcohol-ether and ethylenediamine extraction. It should be noted that all the rat bone samples came from the same powdered pool of Wistar femurs excised from animals of the same age. A vacuum oven was employed to remove the solvents from all samples. Care was taken throughout to insure anhydrous conditions to prevent mineral recrystallization. No measurable change besides a drop in background level was seen in any of the X-ray patterns of the treated bones.

Results and discussion. Fig. 2 shows the validity of the relationship, $I_m/I_{HA} = (1 - W_{am})$, shown as solid line, as evidenced by the linearity of the I_m *vs* percent non-crystalline plot (closed circle). The correctness of the adjustment of the observed relative area (I_b) for the Ca/P ratios, as well as the organic, and water content is shown by the fit of the corrected relative areas to the above plot in Fig. 2 (open circles).

Table II lists results of the determination of percent non-crystalline calcium phosphate in the bone series.

It should be noted that all percent non-crystalline values obtained by the X-ray diffraction method depended upon the choice of a (100% crystalline)) standard hydroxyapatite. If, by chance, the standard material contained some small amount of non-crystalline calcium phosphate then the percent non-crystalline values obtained would have to be adjusted upward. Thus, the "A" percent non-crystalline values reported in Table II (assuming $\mu_{HA} = \mu_{am}$) represent minimum values, thereby reinforcing the view that bone mineral contains a considerable non-crystalline portion. In addition, if the Ca/P ratios and thus the μ values of the non-crystalline apatite phases are unequal, the

TABLE II. Percent of Mineral Phase Found in the Non-Crystalline Form of a Series of Bones.

Sample	% Moisture	% Organic	% Mineral	I_b*	I_m*	Ca/P	% Non-crystalline† A	B
Synthetic hydroxyapatite	—	—	—	3567	3567	1.64	.0	.
Cow bone, untreated	8	34	58	1983	2088	1.65	41.5	44.
Rat bone, "	11	33	56	1943	2106	1.62	41.0	44.
" " , alcohol-ether reflux	11	37	63	1963	2071	1.62	42.0	45.
" " , ethylene diamine reflux	—	—	—	2074	2074	1.62	41.9	45.
" " , alcohol-ether + ethylene diamine reflux	—	—	—	2138	2138	1.62	40.1	43.

* I_b is the uncorrected relative area for whole bone; I_m is the same corrected for the presence of col lagen, water and chemical stoichiometry.

† A, figures calculated assuming $\mu_{HA} = \mu_{am}$; B, figures calculated assuming Ca/P of "HA" and "am' are 1.8 and 1.33, respectively, resulting in $\mu_{HA} = 85.6$ and $\mu_{am} = 76.3$.

percent non-crystalline values will rise if, as in the case of the synthetic experiments, (1), $\mu_{HA} > \mu_{am}$. Column B, Table II, lists the percent non-crystalline values obtained assuming the Ca/P for the non-crystalline phase is 1.33 and the Ca/P for the apatite phase is 1.80, the two extremes reported in a recent chemical analysis of bone (4). The precision of the percent non-crystalline measurement was ± 1%, thus it is reasonable to state that the bones analyzed contained values lying between the extremes represented in columns A and B.

As stated, a standard hydroxyapatite was chosen which yielded a broadened X-ray pattern similar to the patterns of bone apatite. This was done to eliminate the possibility that the non-crystalline X-ray scatter from bone mineral was due to surface ions on the bone apatite crystallites. If it is true that surface ions contribute to the non-crystalline scatter of X-rays, then by choosing a standard with a similar specific surface (using X-ray diffraction line broadening as the criterion) this effect is balanced out and the non-crystalline component observed by the above procedure is truly a second mineral phase.

A secondary result of this experiment worth stressing is that ethylene diamine extraction of bone protein does not alter the mineral phase if anhydrous conditions are maintained throughout the treatment, including elimination of the final water wash.

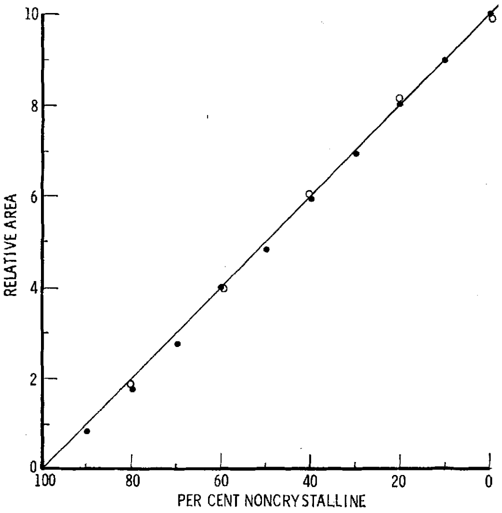

FIG. 2. Closed circles show plot of X-ray diffra tion relative area against per cent of non-crystalline c a series of mixtures of synthetic hydroxyapatite an amorphous calcium phosphate. Open circles represen relative area values of similar mixtures corrected f further admixture of bone organic matrix.

Summary. It was demonstrated by X-ray diffraction that bone mineral from mature cows, humans and Wistar rats, contained a non-crystalline calcium phosphate in additio to the well-known apatite phase. X-ray dif fraction technique was described for deter mination of the percent of this amorphou (non-crystalline) material present in bon mineral, or synthetic mixtures.

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1. Eanes, E. D., Gillessen, I. H., Posner, A. S Nature, 1965, v208, 365.

2. Eanes, E. D., Posner, A. S., Trans. N. Y Acad. Sci., 1965, v28, 233.

3. Alexander, L. E., Klug, H. P., Anal. Chem., 1948, v20, 886.
4. Woodward, H. Q., Health Physics, 1962, v8, 513.

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Histomorphological Changes in Reproductive Organs of Rats Fed Cyclopropenoid Fatty Acids.*† (31074)

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Physiological disturbances involving sexual development and reproduction in the chicken and the rat have been coupled to the ingestion of dietary lipids containing cyclopropenoid fatty acids(1). The effects of cottonseed oil or cottonseed products containing cottonseed oil on sexual development and egg production in the avian species have been extensively reported(1,2). Studies with rats have utilized *Sterculia foetida* oil as a rich source of cyclopropenoid fatty acid(3).

The biologically active fraction of *S. foetida* oil contains sterculic and malvalic acids in a 10 to 1 ratio(2). Sterculic is a C_{19} cyclopropenoid fatty acid whose structure is as follows (4):

$$CH_3-(CH_2)_7-C=C-(CH_2)_7-COOH$$

Malvalic acid has one less C between the cyclopropene ring and the carboxyl end(5).

Schneider(6) reported that daily dietary supplements of 200 mg *S. foetida* oil (30-50% cyclopropenoid fatty acids) fed by capsule to sexually immature pullets starting at 16 weeks of age, impaired ovary-oviduct-uterus and comb development and prevented egg production. When these hens were sacrificed at one year of age, no mature ova were observed in the ovaries. In fertilized eggs from hens fed 50 mg *S. foetida* oil per day, embryonic development ceased after 5 days of incubation (7).

Sexual development of female rats maintained on a 1% safflower-3 % *S. foetida* oil diet from weaning was significantly retarded, estrous cycles were prolonged and irregular, and the reproductive organs (ovaries-oviducts-uteri) were significantly smaller than those of controls(3).

To date, only the gross effects of the cyclopropenoid fatty acids have been reported. This paper reports some of the accompanying histomorphological changes in reproductive tissues as a result of cyclopropenoid fatty acid ingestion.

Methods. Sprague-Dawley female and male rats were maintained from weaning to 6 or 12 months of age on complete purified diets containing either 4% safflower oil, hereafter designated as control, 2% safflower-2% *S. foetida* oil, or 1% safflower-3% *S. foetida* oil, hereafter designated as 2% or 3% *S. foetida* oil diets. Drinking water was provided *ad libitum.* Estrous cycles were characterized by daily vaginal smears taken at approximately the same time each morning by the method of Long and Evans(8). Rats found in proestrus were killed by concussion the next day and the reproductive organs anterior to the cervix were removed, weighed, and fixed in Bouin's fluid. Ovaries and the attached oviducts and mid-portions of uteri were paraffin embedded, sectioned serially at 10 μ and stained with hematoxylin and eosin. Testes and epididymides were excised from *S. foetida* oil and control oil fed rats and were processed in the same manner.

In breeding studies the females and males were maintained in individual cages during

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