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The Reaction of 1-Fluoro-2,4-Dinitrobenzene with Bone

STUDIES ON THE RELATIONSHIP BETWEEN BONE COLLAGEN AND APATITE

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A number of investigators have now utilized FDNB* (Sanger, 1945) to study the reactivity of the ϵ -amino groups of collagen lysine and hydroxylysine in either intact fully mineralized bone or in bone in various stages of demineralization. The reasoning has been that a logical association between the mineral and collagen would be an interaction between the positively charged ϵ -amino groups and the negatively charged phosphate ions of the hydroxylapatite.

Solomons & Irving (1955) first described experiments in which the number of reactive ϵ -amino groups in collagen of hard tissues were estimated with FDNB during decalcification *in vitro*. They reported a linear relationship between the release of mineral and the reactivity of the ϵ -amino groups and suggested that this was evidence for a bonding between these groups and apatite. These results were amplified (Solomons & Irving, 1956, 1958) by comparison of the reactivity of collagens from hard and soft tissues. Under conditions of simultaneous demineralization and treatment with FDNB nearly all the ϵ -amino groups of hard tissues reacted, compared with only two-thirds in soft tissues.

Glimcher (1960, 1961), presenting data on bone of various densities, suggested that the extent of the reaction of the ϵ -amino groups of mineralized bone with FDNB was limited by diffusion (i.e. governed by the degree of occlusion of the collagen

by apatite crystals). Demineralization would thus remove the diffusion barrier and permit FDNB to react with the ϵ -amino groups of collagen.

Cartier & Lanzetta (1961), however, reported that 53 % of the total ϵ -amino groups of collagen in finely ground ox bone reacted with FDNB independently of the degree of demineralization until 97 % removal of the mineral. Beyond this stage an additional 22 % reacted, giving a total of 75 % of the lysine and hydroxylysine residues in bone collagen. The lack of effect of demineralization on the reaction with FDNB, except at almost complete removal of bone salt, contra-indicated a diffusion phenomenon.

The present studies were undertaken to clarify these apparently contradictory results and to elucidate the nature of the relationship between the mineral phase and the ϵ -amino groups of bone collagen. Our objectives were: (1) to test the reactivity of the ϵ -amino groups of bone collagen with FDNB in fully demineralized bone and at various stages of demineralization; (2) to evaluate the effect of diffusion on the rate and extent of the reaction of the ϵ -amino groups of bone collagen with FDNB and different particle sizes of bone. The result of these first experiments showed that it was necessary to test the reactivity of the ϵ -amino groups of bone collagen with FDNB in intact fully mineralized bone in an anhydrous reaction system in which hydrolysis of FDNB was kept at a minimum.

* Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

MATERIALS AND METHODS

Preparation of bone samples

One metatarsus from a freshly slaughtered Holstein cow was quickly freed of periosteal tissue, sawn lengthwise and the marrow rinsed out by a jet of hot water. The epiphyseal ends were sawn off and the dense cortical portion of the diaphysis was freed of any cancellous bone, rapidly frozen by immersion in a freezing bath of isopentane chilled by solid CO₂-ethanol mixture, split into smaller pieces with a mallet and surgeon's bone chisel and then ground with solid CO₂-ethanol to pass a 10-mesh screen in a micro-Wiley mill prechilled with solid CO₂. After grinding, the bone particles were sieved to various ranges of particle size by immersion in a large volume of water and rinsing the suspension through a series of graded sieves. The particles collected were 10-20, 20-40, 40-60, 60-80, 80-100, 100-200, 200-325 and < 325 mesh size (U.S. Standard Sieve Series, 1940). These sieved particles were drained and blotted on soft cellulose tissue, resuspended in a small volume of aq. 70% ethanol, layered over with isopentane, sealed and stored at -25° until required. When needed, the particles were suspended twice in diethyl ether-ethanol (1:1, v/v) to remove any remaining lipids, rinsed with aq. 95% ethanol, blotted dry with soft cellulose tissue and weighed. For the anhydrous experiments, the bone was placed in absolute ethanol overnight before treatment. The chemical composition of this carefully prepared bone is presented in Table 1. Material from this single bone was used in all of the experiments reported.

Treatment with 1-fluoro-2,4-dinitrobenzene

The details of the treatment procedures with FDNB are presented in Table 2. However, series 1 and 2 were the same in pH (8.0), sodium hydrogen carbonate concentration (0.67%), ionic strength (0.5M-EDTA $\equiv$ 1.05M-sodium acetate, by conductivity) and organic functional group of the buffer salt (acetate), to compare specifically the effect of decalcification on the FDNB reaction. Series 4 was performed in absolute ethanol to prevent hydrolysis of FDNB and recrystallization of the bone mineral during treatment, a problem encountered in series 2 and 3. In all four series of treatments, the effect of particle size (i.e. surface area and diffusion distance) was related to the rate and extent of the reaction of FDNB with bone collagen. For treatment times

of more than 24 hr., solutions were changed daily for the first 3 days and thereafter at the end of each week. After treatment the insoluble materials were thoroughly rinsed with solvents appropriate to remove unchanged FDNB, dinitrophenol and other contaminants, avoiding water with the anhydrous specimens. The particles were weighed after drying overnight at 60° in a vacuum oven and cooling in a desiccator over anhydrous CaCl₂.

Chemical analysis

In series 1 and 2, all of the samples were hydrolysed with constant-boiling HCl (6N) at 105° for 16 hr. in sealed glass tubes. The hydrolysates were filtered to remove humin and diluted appropriately. In addition to the determination of ϵ -DNP-amino acids, nitrogen, calcium, phosphate and fluoride analyses were performed on the hydrolysate of each bone sample. Because some fluoride was lost during hydrolysis, in series 3 and 4, samples of each particle size and treatment time were ashed for fluoride analysis and ash content, and others were hydrolysed in acid for analyses of the other chemical constituents. With the initial samples, the acidic hydrolysates were extracted with ether in an attempt to remove α -DNP-amino acids. However, since none was found, the extracting step was eliminated in subsequent samples.

The ϵ -DNP-lysine and hydroxylysine content of FDNB-treated bone was determined by measuring the extinction of the diluted hydrolysates at 360 m μ against a blank of similarly diluted non-treated bone hydrolysates. The amount of tyrosine in collagen is so small that the interference from its O-DNP derivative at 360 m μ was insignificant. A factor of $\mu\text{moles}/0.97$ was applied to correct for the destruction (3%) of the ϵ -DNP-lysines during the 16-hr. hydrolysis.

The amount of collagen in each bone sample was determined by nesslerization of H₂SO₄ digests of samples of the diluted hydrolysate. Ammonium chloride was used as a standard and collagen was calculated as N $\times$ 5.55.

The inorganic phosphate content of the hydrolysates was analysed by the Martin & Doty (1949) modification of the Berenblum & Chain (1938) method.

Calcium was determined initially by the Williams & Wilson (1961) method and subsequently by a modification of the method developed in this Laboratory which involved a different extracting agent and the stabilization of the coloured complex in anhydrous alkaline methanol. This

Table 1. *Chemical composition of untreated ox cortical bone*

Percentage composition is expressed on the basis of dry fat-free weight. Values are means $\pm$ S.E.M.

Constituent	No. of samples	Percentage
Ash	24	65.58 $\pm$ 0.13
Collagen (N $\times$ 5.55)	24	22.17 $\pm$ 0.15
Calcium	24	24.92 $\pm$ 0.09
Phosphorus	24	11.89 $\pm$ 0.04
Fluorine	14	0.061 $\pm$ 0.004
Constituent ratio		Ratio
Phosphorus/calcium (molar)	30	0.617 $\pm$ 0.002
Phosphorus/collagen (m-moles/g.)	30	17.30 $\pm$ 0.09
Calcium/collagen (m-moles/g.)	30	28.11 $\pm$ 0.17
Fluoride/collagen (m-moles/g.)	14	0.145 $\pm$ 0.009

modification gave greatly improved colour stability and was not affected by tenfold molar excesses of phosphate, F^- , citrate or Mg^{2+} ions. The method was as follows.

Reagents. Colour reagent: 0.4% (w/v) glyoxal bis-(2-hydroxyanil) in anhydrous A.R. methanol, prepared fresh daily.

Developing solution: 10.0 g. of NaOH + 0.5 g. of Na_2CO_3 in 100 ml. of deionized water.

Extracting agent: benzene-*n*-hexanol (1:1, v/v) (A.R. materials).

Preserving solution: anhydrous methanol made to 0.02N with dry NaOH was used. The methanolic solution had to be absolutely anhydrous to prevent the colour from fading.

Procedure. Preserving solution (4 ml.) was placed in separate colorimeter tubes. The tubes were stoppered and set aside.

In glass-stoppered 10–25 ml. test tubes were placed 2.0 ml. of aqueous sample containing 2–20 $\mu g.$ of calcium and 1.0 ml. of colour reagent, with brief mixing. The next steps were done in groups of six to eight tubes each and had to be completed within 15–20 min. after addition of the developing solution because the calcium-glyoxal complex was unstable in aqueous media.

To each tube was added 0.2 ml. of developing solution and the contents of the tubes were mixed briefly. Next, 3.0 ml. of extracting agent was added, and the tubes were tightly stoppered and shaken vigorously 40 times with formation of an unstable emulsion.

The tubes were then centrifuged at 2500 rev./min. for 1 min. A sample (1.0 ml.) of the reddish supernatant was pipetted into the colorimeter tubes containing the an-

hydrous preserving solution and the contents of the tubes were mixed.

The tubes were read at 535 $m\mu$. The colour was stable for at least 24 hr. if the tubes were tightly stoppered. The blank gradually turned pale yellow, but this did not interfere with the readings at 535 $m\mu$. The standard deviation of the method was 3%.

Fluoride analyses of the hydrolysates were performed with the Singer & Armstrong (1959) micro-distillation apparatus and the Megregrian (1954) photometric method. Fluoride analysis of the ashed samples was done mainly by the 4,5-dihydroxy-3-(*p*-sulphophenylazo)naphthalene-2,7-disulphonic acid method of Wharton (1962). However, for specimens with very high fluoride content, the thorium chloroanilate method of the Fisher Scientific Co. was used (see *Technical Data Sheet* no. TD-138; Fisher Scientific Co., Medford 55, Mass., U.S.A.). The separation of fluoride from interfering ions in the ashed specimens was accomplished by HF diffusion in sealed polyethylene chambers.

X-ray diffraction was carried out on a Norelco diffractometer, with a scanning speed of $\frac{1}{4}^\circ 2\theta/\text{min.}$ for maximum resolution. The radiation was $Cu K_\alpha$ at 15 ma and 35 kv.

RESULTS

Simultaneous treatment with 1-fluoro-2,4-dinitrobenzene and demineralization with EDTA (series 1)

In contrast with previous findings (Solomons & Irving, 1958), the relationship between demineralization and dinitrophenylation was not strictly

Table 2. *Treatment procedures with 1-fluoro-2,4-dinitrobenzene*

All treatments were performed at room temperature (22–23°).

Series no.	Particle sizes employed (mesh)	Treatment times (hr.)	No. of samples of each time and size	Wt. of sample (mg.)	Treatment soln.	Purpose of treatment
1	10–20, 20–40, 100–200	6, 24, 72, 168, 336 and 720	2	200	5 ml. of ethanolic 10% FDNB + 15 ml. of 0.5M-EDTA containing 0.67% of $NaHCO_3$ (pH 8.0)	Test reactivity of amino groups to FDNB at various stages of demineralization
2	10–20, 20–40, 100–200	6, 24, 72, 168, 336 and 720	2	200	5 ml. of ethanolic 10% FDNB + 15 ml. of 1.05M-sodium acetate containing 0.67% of $NaHCO_3$ (pH 8.0)	Test effect of diffusion on rate and extent of FDNB reaction with non-decalcified bone
3 (a)	60–80	6, 24, 48, 168, 336, 744 and 1176	4	100	10 ml. of ethanolic 5% FDNB + 5 ml. of 2% $NaHCO_3$	Repeat work of Cartier & Lanzetta (1961) and Glimcher (1960, 1961). Determine maximum reaction of FDNB with bone collagen without decalcification
(b)	<325	60				
4 (a)	60–80	6, 24, 48, 168, 336 and 744	4	125	20 ml. of ethanolic 5% FDNB	Test reactivity to FDNB of ϵ -amino groups of 'intact' mineralized bone in anhydrous medium
(b)	10–20, 20–40, 40–60, 60–80, 80–100, 100–200	720				
(c)	200–325, <325	60				

linear (Fig. 1). The extent of the reaction of FDNB with collagen was closely related to the degree of demineralization until most of the mineral was removed, but at least one-third of the FDNB reaction took place after 90% demineralization. The rate of demineralization of the 100–200-mesh particles was so rapid that the FDNB reaction did not always keep pace. The maximum extent of dinitrophenylation was 275 ± 4 (S.E.M.) μ moles/g. of collagen, which is 78.5% of the total present (350 μ moles/g.; Eastoe, 1955). Although this value is considerably less than 330 μ moles/g. (Solomons & Irving, 1958), it is only 4% higher than the 264 μ moles/g. reported by Cartier & Lanzetta (1961). A probable explanation for the apparent discrepancy between the three figures is found in the factor applied to correct for the losses of the ϵ -DNP-lysines during hydrolysis. Porter & Sanger (1948), Cartier & Lanzetta (1961) and the present authors have all found approximately 97% recovery of free ϵ -DNP-lysine when hydrolysed with protein at 105° with 6N-hydrochloric acid for 16 hr. As Cartier & Lanzetta (1961) have observed, the 80% recovery reported by Solomons & Irving (1958) seems to be unreasonably low. The difference between Cartier & Lanzetta's (1961) value and

that of the present paper probably arises from the fact that we have used the 0.97 correction factor, whereas Cartier & Lanzetta (1961) did not use any at all.

Treatment with 1-fluoro-2,4-dinitrobenzene in a non-decalcifying sodium acetate system (series 2)

When the bone particles were treated according to series 2 (Table 2) decreasing the particle size increased the rate of the dinitrophenylation reaction (Fig. 2), but even with the 100–200 mesh particles the reaction was slow and a constant value for DNP-amino groups/g. of collagen was not attained by 30 days. The failure readily to achieve equilibrium suggested that the rate of the FDNB reaction with bone collagen was not a simple diffusion-limited process. The shape of the FDNB-reaction curves (Fig. 2) was similar to that obtained for the exchange of radioactive phosphate in bone (Neuman & Mulryan, 1950), for the uptake of fluoride by apatite (McCann & Bullock, 1957), or for the uptake of radioactive calcium by bone powder (Dallemagne, Bodson & Ueten, 1958; Richelle, 1961). These authors have all interpreted the failure to achieve steady-state rapidly to be evidence for recrystallization. Therefore recrystal-

Fig. 1. Relationship between reactivity of ϵ -amino groups of bone collagen with FDNB to the stage of demineralization. Bone particles (200 mg.) were treated with 5 ml. of ethanolic 10% FDNB + 15 ml. of 0.5M-EDTA soln. (pH 8.0) containing 0.67% of NaHCO_3 . O, 10–20 mesh particles; ●, 20–40 mesh particles; ▲, 100–200 mesh particles.

lization of the apatite appeared to be responsible for the gradual increase in reactive ϵ -amino groups after the initial rapid reaction.

The highest figure obtained for the dinitrophenylation of non-demineralized bone with this system was $145 \mu\text{moles/g.}$ of collagen (41 % of the total lysine residues). Using much more finely ground bone, Cartier & Lanzetta (1961) obtained a higher constant value of $186 \mu\text{moles/g.}$ of collagen for non-demineralized and partially demineralized bone. Inasmuch as the FDNB reaction in our experiments was not complete, the higher value of Cartier & Lanzetta (1961) seems reasonable.

Chemical and physical analysis of the aqueous-FDNB-treated bone particles showed that the composition of the bone mineral was considerably altered by the FDNB treatment. The chemical changes were a large loss of phosphate, a very large increase in fluoride, but very little change in calcium (Fig. 3). Because the phosphate and fluoride content of the bone changed markedly during the course of the treatment, the concentration of the mineral constituents are given as m-moles/g. of bone collagen. The initial values for calcium and phosphate were 28.11 ± 0.17 (S.E.M.) and 17.30 ± 0.09 m-moles/g. of collagen respectively.

Within 6 hr. after treatment, a 4–6 % loss of both calcium and phosphate occurred. These losses did not alter the phosphorus/calcium ratios significantly but did cause a small decrease in the phosphorus/collagen and calcium/collagen ratios. No further calcium loss was observed during the complete 30-day treatment. The phosphate values, however, declined to 78.6, 70.5 and 39.3 % of the original phosphate for the 10–20, 20–40 and 100–200 mesh bone samples respectively. The combination of phosphate loss and unchanged calcium resulted in decreases in the phosphorus/calcium molar ratios to values as low as 0.25, the theoretical ratio for apatite being 0.60 (Fig. 4).

As stated previously, there was a large increase in the fluoride content of bone as a result of the treatment with FDNB. Ashed untreated bone contained 0.145 ± 0.009 m-mole of fluoride/g. of collagen (0.061 % of fluoride, dry wt.). The hydrolysates of the untreated bone, however, gave a value of only 0.051 % of fluoride (dry wt.), indicating a considerable loss of fluoride during acid hydrolysis. Nevertheless, hydrolysates of the FDNB-treated bone had ratios as high as 29.4 m-moles of fluoride/g. of collagen (Fig. 3) or 1.1 moles of fluoride/mole of calcium (Fig. 4). Since the

Fig. 2. Effect of demineralization on rate and extent of reaction of FDNB with bone collagen: comparison of matched demineralizing and non-demineralizing FDNB-treatment systems. Bone particles (200 mg.) were treated with 5 ml. of ethanolic 10% FDNB + 15 ml. of either 0.5M-sodium EDTA soln. (---) or 1.05M-sodium acetate soln. (—), both pH 8.0, and with the same ionic strength (by conductivity), NaHCO_3 concentration (0.67%) and organic functional groups of the buffer (acetate). O, 10–20 mesh particles; ●, 20–40 mesh particles; ▲, 100–200 mesh particles.

Fig. 3. Effect of treatment with aq. sodium acetate-FDNB on chemical composition of bone. Chemical constituents are expressed as ratios to the collagen content of bone because mineral composition changed during treatment. - - - -, Calcium/collagen; —, P/collagen; - · - · -, F/collagen. ○, 10-20 mesh particles; ●, 20-40 mesh particles; △, ▲, 100-200 mesh particles.

Fig. 4. Change in ratios of mineral constituents of bone during treatment with aq. sodium acetate-FDNB. Phosphorus and F contents are expressed as molar ratios to Ca, as the Ca content remained essentially unchanged during treatment. - - - -, Theoretical molar ratios of apatite and fluorapatite; —, P/Ca; - · - · -, F/Ca. ○, 10-20 mesh particles; ●, 20-40 mesh particles; △, 100-200 mesh particles.

Table 3. *Changes in chemical composition of 60–80 mesh bone particles treated for various periods of time with 1-fluoro-2,4-dinitrobenzene in aq. 67% ethanol or in absolute ethanol (series 3a and 4a)*

Treatment time (days)	Percentage					Molar ratio		Percentage		ϵ -DPN-amino acids (μ moles/g. of collagen)
	Ash	Collagen	P	Ca	F	P/Ca	F/Ca	Fluor-apatite	Calcium fluoride	
None	66.0	22.6	11.6	25.1	0.06	0.596	0.005	2.7	0	0
Untreated										
Treated with FDNB in aq. 67% ethanol (series 3)										
0.25	66.3	22.0	11.8	25.8	0.74	0.590	0.060	30.2	0	47.6
1	66.3	22.6	11.6	25.6	1.24	0.584	0.102	50.1	0	68.5
2	66.0	23.3	11.8	26.4	1.75	0.579	0.140	70.0	0	88.6
7	62.2	23.2	10.6	24.7	2.32	0.556	0.198	99.0	0	118.8
14	65.2	23.8	11.1	26.4	3.02	0.545	0.242	97.6	2.4	129
21	65.6	25.6	10.8	26.6	6.78	0.528	0.539	81.2	18.8	175
31	70.5	24.3	8.9	22.2	10.80	0.516	1.030	60.4	39.6	186
49	62.7	24.3	6.8	25.0	13.55	0.353	1.145	47.5	52.5	240
Treated with FDNB in absolute ethanol (series 4)										
0.25	66.2	23.3	11.6	25.5	0.14	0.590	0.012	5.8	0	39.2
1	66.4	23.0	11.9	26.2	0.30	0.588	0.024	11.9	0	65.4
2	65.5	23.6	12.0	25.4	0.45	0.603	0.037	18.6	0	73.7
7	66.6	23.0	11.8	24.8	1.08	0.618	0.092	45.9	0	96.3
14	69.6	23.2	12.6	27.2	1.08	0.600	0.115	57.5	0	102.5
21	65.0	23.6	11.7	25.0	1.72	0.608	0.145	72.5	0	92.5
31	66.5	23.6	11.9	26.0	2.09	0.595	0.171	85.5	0	110.0

fluoride/calcium molar ratio of pure fluorapatite is only 0.20, more fluoride was present than could be accounted for on the basis of fluorapatite alone. Comparison of the phosphorus/calcium and fluoride/calcium ratios in Fig. 4 also showed that the large decrease in the first ratios occurred only after the second ratios exceeded 0.20 and then progressed in direct proportion to the increase in fluoride.

Direct evidence that fluoride was altering the apatite structure was obtained from X-ray-diffraction-powder patterns of the FDNB-treated bone. The presence of a large quantity of poorly crystalline calcium fluoride was shown and there was evidence that the crystallinity (i.e. crystal size or crystal perfection or both) of the remaining apatite phase was increased (Fig. 7).

Treatment with 1-fluoro-2,4-dinitrobenzene in aq. 67% ethanol (series 3)

Less-marked changes in mineral composition were observed with the series 3 system (Table 3). The X-ray-diffraction-powder patterns nevertheless revealed an initial increase in the crystallinity of the apatite and later the appearance of a poorly crystalline calcium fluoride phase (Fig. 5). This finding confirms the improvement in crystallinity observed in bones with increasing fluoride content reported by Zipkin, Posner & Eanes (1962).

Attempts were made to determine the maximum extent of the FDNB reaction with bone collagen by using this reaction system. When 60–80 mesh bone was used, despite reaction times as long as 7 weeks, no constant value was attained (Table 3).

The highest figure obtained was 240 μ moles/g. of collagen. However, this amount appeared to represent not only apatite destruction, but also an alteration of the collagen itself because of the prolonged treatment. Therefore, in an attempt to speed the reaction, < 325 mesh bone was employed. Although it reacted rapidly and gave a value 174 ± 3 μ moles/g. of collagen between 2.5 and 14 days of treatment, it was found to be altered in its reactivity to FDNB with the anhydrous treatment of series 4 (Table 2). Because of these difficulties, no exact figure can be reported for the maximum extent of reaction when all of the apatite was dismutated to calcium fluoride. However, other evidence suggests that the limit should be in the range 175–195 μ moles/g. If the linear portion of the curve in Fig. 1 (series 1) was extrapolated to complete demineralization, a range 175–190 μ moles/g. of collagen was obtained. Similarly, if the extent of FDNB reaction with collagen (series 2) was plotted against the phosphorus/collagen ratio (Fig. 6), extrapolation to complete loss of phosphate gave a value 185–195 μ moles/g. These values are in close agreement with that (186 μ moles/g.) obtained by Cartier & Lanzetta (1961) using non-demineralized and partially demineralized cow bone.

Hydrolysis of 1-fluoro-2,4-dinitrobenzene in aqueous systems

An attempt was made to explain the large amounts of fluoride present in treated bone, which exceeded that expected from the reaction of FDNB

with collagen by as much as 100-fold. The consistent observation that dinitrophenol rapidly appeared after the addition of the aqueous buffers to the ethanolic FDNB solutions led us to analyse the reaction mixtures for free F^- ion. The reaction system of series 2 (aq. 25% ethanol) hydrolysed sufficient FDNB by 24 hr. to yield 465 p.p.m., by 48 hr. 750 p.p.m. and by 1 week 1900 p.p.m. of free F^- ion. Similarly, the reaction system of series 3 (aq. 67% ethanol) used by Glimcher (1960, 1961) and Cartier & Lanzetta (1961) yielded 420 p.p.m. of free F^- ion after 24 hr. Any of these concentrations of F^- ion were more than sufficient to dismutate apatite to calcium fluoride (McCann & Bullock, 1955). In view of these findings, previous interpretations of the reactivity of the ϵ -amino groups of

bone collagen to FDNB must be evaluated again. The FDNB reaction mixtures generally employed to treat proteins contain water, which hydrolyses FDNB to such an extent that the F^- ion released undoubtedly caused at least partial dismutation and extensive recrystallization of the mineral phase of the treated bone.

*Treatment with 1-fluoro-2,4-dinitrobenzene
in absolute ethanol (series 4)*

Because of the considerable alteration in the chemical and physical character of the bone mineral during treatment with aqueous FDNB reagents, an anhydrous system was employed. In Tables 3 and 4 are reported the changes in chemical composition of bone particles after various treat-

Fig. 5. X-ray diffraction patterns of bone showing progressive alteration of the apatite mineral structure during treatment with FDNB. Patterns are from (a) untreated 60–80 mesh bone and this bone treated for (b) 7, (c) 14, (d) 21, (e) 31 and (f) 49 days with FDNB in aq. 67% ethanol: (series 3, Table 2). Numbers above the diffraction peaks are Miller indices of the crystal-lattice planes. Patterns of bone treated 0–21 days reveal a progressive narrowing and increased resolution of the 211, 112, 300 and 202 diffraction peaks of apatite, indicating a progressive increase in the crystallinity of the apatite. Beyond 21 days the crystallinity and amount of apatite decreases. Patterns of samples treated 14 days or more show the appearance and increase in size of the 111 peak of calcium fluoride. By 49 days, calcium fluoride becomes the dominant phase. For corresponding chemical changes and the extent of dinitrophenylation of the bone collagen, see Table 3.

ment times. No alteration in phosphorus/collagen or calcium/collagen ratios or in phosphorus/calcium ratios occurred. The reaction of FDNB with collagen in 60–80 mesh bone (Table 3) reached equilibrium by 7 days; however, the fluoride content of the bone continued to increase throughout the treatment period.

To test the effect of particle size with the anhydrous system, a graded series of bone particles was employed. The results revealed that particle size had minimal influence on the reaction except for the very finely ground < 325 mesh fraction (Table 5). All phosphorus/calcium ratios were similar, as were the fluoride/calcium ratios, except

Fig. 6. Relation of phosphate loss to reaction of FDNB with bone collagen in the aq. sodium acetate–ethanol (75:25) reaction described in Table 2. ○, 10–20 mesh particles; ●, 20–40 mesh particles; △, 100–200 mesh particles.

Table 4. Chemical composition of non-demineralized bone, untreated and treated with 1-fluoro-2,4-dinitrobenzene in absolute ethanol (series 4b and c)

Percentage composition is expressed on the basis of dry fat-free weight. Values are means±s.e.m. Particle sizes ranged from 20–40 mesh to 200–325 mesh; see Tables 2 and 5.

Constituent	Untreated samples		FDNB-treated samples	
	No.	Percentage	No.	Percentage
Ash	12	65.58±0.25	12	65.58±0.06
Collagen	12	22.33±0.25	12	22.00±0.16
Calcium	12	25.11±0.10	12	24.74±0.13
Phosphorus	12	11.91±0.03	12	11.85±0.07
Fluorine	12	0.059±0.004	12	2.19±0.05

Constituent ratios	Untreated samples		FDNB-treated samples	
	No.	Ratio	No.	Ratio
Ca/collagen (m-moles/g.)	12	27.97±0.26	12	28.04±0.27
P/collagen (m-moles/g.)	12	17.24±0.16	12	17.40±0.16
F/collagen (m-moles/g.)	12	0.141±0.010	12	5.25±0.12
ε-DNP-amino acids/collagen (μmoles/g.)	12	0	12	93.3±2.0
P/Ca (molar)	12	0.615±0.001	12	0.620±0.004
F/Ca (molar)	12	0.0050±0.0003	12	0.187±0.004

for the two finest-mesh sizes, which were treated for only 2.5 days. Likewise only small variations appeared between particle sizes in the extent of FDNB reaction with collagen, with the exception of the < 325 mesh samples. Here the fine grinding probably altered the collagen-apatite linkage and permitted the increased reaction.

X-ray diffraction patterns of the 30-day samples (Fig. 7*d*) revealed only a barely detectable increase in the crystallinity of the treated bone apatite. No evidence for calcium fluoride was found. We

have found that 5–10% of calcium fluoride in synthetic hydroxylapatite can be easily detected by the X-ray-diffraction method used. Similarly, data presented in Fig. 5 and Table 3 indicate that 2.5% of calcium fluoride can be detected. Nevertheless, these X-ray findings did not completely exclude the presence of calcium fluoride. However, if it was present it was either in extremely small amounts or in a very poorly crystalline form. Provided that there was absence of calcium fluoride, the fluoride/calcium ratios (which nearly

Table 5. *Effect of particle size on the chemical composition of bone treated with ethanolic 5% 1-fluoro-2,4-dinitrobenzene (series 4b and c)*

Particle size (mesh)	Period of treatment (days)	P/Ca ratio (molar)	F/Ca ratio (molar)	Fluorapatite (%)	ϵ -DNP-amino acids (μ moles/g. of collagen)
10–20	30	0.608	0.195	97.5	74.5
20–40	30	0.602	0.182	91.0	80.7
40–60	30	0.638	0.196	98.0	91.8
60–80	30	0.628	0.181	90.5	93.3
80–100	30	0.618	0.190	95.0	96.2
100–200	30	0.630	0.167	83.5	87.8
200–325	2.5	0.575	0.027	13.5	90.2
<325	2.5	0.607	0.050	25.0	140.3

Fig. 7. Effect of water in FDNB reaction mixture on X-ray-diffraction-powder patterns of FDNB-treated bone. (a) Untreated bone; (b) bone treated with 2.5% FDNB in aq. 25% ethanol (series 2, Table 2); (c) bone treated with 3.3% FDNB in aq. 67% ethanol (series 3, Table 2); (d) bone treated with 5.0% FDNB in absolute ethanol (series 4, Table 2). Numbers above peaks are Miller indices of crystal-lattice planes. Note increased resolution of the 211, 112, 300, 202 complex of (b). Also note the appearance of the CaF_2 111 peak in (b) and (c). Narrower and more resolved peaks indicate a more crystalline apatite (Zipkin, Posner & Eanes, 1962). Only faintly increased crystallinity is observed after anhydrous FDNB treatment (d) and no CaF_2 is detected. Samples were treated for 30 days.

attained the theoretical value of 0.2 for pure fluorapatite; Table 5) indicated a practically complete penetration of the bone particles by FDNB. This was supported by the fact that particle size had minimal effect either on the extent of the FDNB reaction with the collagen or on the fluoride content of the treated samples (Table 4). The minimal influence of particle size further suggested that diffusion was not a major factor in the extent of the FDNB reaction under these conditions. At equilibrium this value averaged $93.3 \pm 2.0 \mu\text{moles/g.}$ of collagen for particle sizes ranging from 20–40 to 200–325 mesh (Table 5). This amount was slightly more than 25 % of the total ϵ -amino groups in collagen and was only half the value ($186 \mu\text{moles/g.}$) found by Cartier & Lanzetta (1961) with aqueous treatment.

DISCUSSION

The effects expected by varying particle size were in the surface area and, probably of more importance, the diffusion distance. It was not surprising therefore that the observed effect of decreasing the particle size was primarily an increase in the rate of the reaction, regardless of whether demineralization or dinitrophenylation was considered. The extent of the reaction, on the other hand, did not seem to be affected by particle size when equilibrium was attained, the exception being the very finely divided samples prepared to repeat the work of Cartier & Lanzetta (1961) mentioned previously.

The second variable tested was the effect of the degree of demineralization. Although the process of demineralization in general increased the rate of the FDNB reaction, the fact that a sizable portion of the reaction occurred only in the final states of demineralization clearly indicated that more was involved than simple removal of a diffusion barrier.

The third variable tested (the effect of the water content of the FDNB reaction mixture) was found to have marked effects on the reactivity of bone to FDNB. In the first place, FDNB was only very slightly soluble in water and in the aq. 25 % ethanol systems a considerable portion separated as a heavy oily phase, greatly reducing its effective concentration. Further, because FDNB hydrolysed very readily on exposure to water, high concentrations of F^- ions rapidly built up and were sequestered by the bone mineral, markedly increasing its crystallinity. This increase in crystallinity indicated that extensive recrystallization had occurred. With further increases in fluoride content, the apatite dismutated to calcium fluoride and soluble orthophosphate that was leached from the bone when sufficient water was present.

The solvation of ions also appears to play a role in the reaction of the ϵ -amino groups with FDNB.

In contrast with the anhydrous-FDNB reactions, which attained equilibrium after a relatively small portion of the free amino groups had reacted, the aqueous-FDNB reactions failed to attain equilibrium and continued to increase in direct proportion to the extent of apatite recrystallization and dismutation. A reasonable explanation for this difference is that FDNB cannot react with ϵ -amino groups electrovalently bonded to the apatite lattice unless water is present both to dissociate the H^+ ion from the cationic amino group and to permit ion mobility not present in the solid crystalline state. The reactivity of amino groups in non-aqueous media can be explained if they are not directly involved in any linkages, although it is possible that residual bound water may have permitted a portion of these amino groups to react with FDNB. Residual water probably did account for the barely detectable recrystallization which occurred in the anhydrous-FDNB-treated bone.

These data offer evidence that varying degrees of bonding exist between the mineral and the ϵ -amino groups of bone. On the basis of their reactivity to FDNB, the ϵ -amino groups have been divided into four categories. The most reactive groups (amounting to about 25 % of the total in collagen), which react with FDNB under anhydrous conditions, do not appear to be directly associated with the apatite. The second category of ϵ -amino groups (amounting to 25–30 % of the total), which react with FDNB in direct proportion to the alteration of the apatite, are very probably electrovalently bonded to phosphate ions in the apatite.

The third category of ϵ -amino groups, comprising another 25 % of the total, appear to be more strongly bonded to the mineral and react with FDNB only after demineralization is almost complete. The linkage appears to be labile to acid demineralization (Cartier & Lanzetta, 1961) or to EDTA chelation of calcium, whereas it does not appear to be disrupted by dismutation or recrystallization of the apatite. These ϵ -amino groups may be bonded to pyrophosphate and involved in apatite nucleation (Cartier & Lanzetta, 1961); however, more direct evidence is needed to substantiate this hypothesis.

The fourth category of ϵ -amino groups (approximately 21 % of the total), which do not react with FDNB even after complete demineralization, are likely to be involved in covalent cross-linkages within the collagen molecule. However, they may be sterically inaccessible within the helices of the collagen in its undenatured state. More reactive systems may react with the total amount (Glimcher, 1961); however, we have found, like Cartier & Lanzetta (1961), that a consistent fraction of the total ϵ -amino groups of bone fails to react with FDNB.

It is difficult to predict how variable the size of these fractions of ϵ -amino groups may be. The state of mineralization or the age of the bone probably affects the proportion in each category.

SUMMARY

1. The reaction of 1-fluoro-2,4-dinitrobenzene with ox cortical bone has been investigated under various experimental conditions designed to permit evaluation of the nature and extent of the relationship between the bone matrix and bone mineral. The effect of particle size of bone, the state of mineralization and the water content of the 1-fluoro-2,4-dinitrobenzene reaction medium were tested on the rate and extent of the dinitrophenylation of bone collagen.

2. Decreasing particle size increased the rate of the dinitrophenylation reaction with collagen, but did not affect the extent of the reaction if steady state was attained.

3. The extent of the 1-fluoro-2,4-dinitrobenzene reaction with bone collagen was directly proportional to the extent of demineralization until nearly all of the mineral was removed. In the final stages of demineralization an additional 20–25 % of the total ϵ -amino groups of bone collagen reacted with 1-fluoro-2,4-dinitrobenzene.

4. The mineral of bone in aqueous-1-fluoro-2,4-dinitrobenzene treatment procedures underwent extensive recrystallization and dismutation. The extent of dinitrophenylation of the bone collagen was directly related to the degree of alteration of the mineral structure.

5. When alteration of the mineral structure was prevented by treating bone with 1-fluoro-2,4-dinitrobenzene under anhydrous conditions, only 25 % of the total ϵ -amino groups of bone collagen reacted with 1-fluoro-2,4-dinitrobenzene.

6. These data suggest that nearly half of the total ϵ -amino groups of fully mineralized bone are not directly associated with the mineral. Twenty-five per cent of the total appears to remain free, whereas 20–25 % is internally bonded within the collagen molecule. The remaining 45–50 % of the ϵ -amino groups appears to be directly linked to the mineral. Of these, half seem to be associated by

electrovalent attraction, probably to orthophosphate ions in apatite. The other half seem to be more strongly bonded to the mineral, perhaps to pyrophosphate ions, as Cartier & Lanzetta (1961) have suggested.

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