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# CHARACTERISTICS OF INSOLUBLE PROTEIN OF TOOTH AND BONE - I. FLUORESCENCE OF SOME ACIDIC HYDROLYTIC FRAGMENTS

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# CHARACTERISTICS OF INSOLUBLE PROTEIN OF TOOTH AND BONE—I

## FLUORESCENCE OF SOME ACIDIC HYDROLYTIC FRAGMENTS*

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**Summary**—Peptides were prepared by partial acid hydrolysis of insoluble protein of tooth and bone. Cellulose column electrophoresis was employed to separate major fluorescing peptides. Using parent gelatins, peptide mixtures, and major peptides fractions of the calcified proteins the following measurements were taken: u.v. absorption and fluorescent excitation and emission spectra, extinction coefficients and total fluorescence. There is evidence that tryptophan and tyrosine contribute to the material of parent gelatin which absorbs at 280 mμ, and are partially responsible for the fluorescence of the preparations studied here. The total fluorescence of calcified proteins occurs as a combination of excitation of three or more fluorescing molecules, tyrosine and tryptophan to the extent of their absolute concentration and another fluorophoric group(s) presently unidentified.

### INTRODUCTION

THAT teeth fluoresce when irradiated with ultraviolet light has long been known (STUBEL, 1911). The phenomenon has been investigated with varying conclusions as to the substance or substances responsible for fluorescence. TIEDE and CHOMSE (1934) were able to combine native collagen and tricalcium phosphate at 300°C and produce the typical blue-white fluorescence of tooth and bone. When hydrogen peroxide and ammonia destroyed fluorescence they indicated that the organic portion was the fluorescing component. In contrast, GLASSER and FONDA (1938), after presumably burning out the organic content of enamel at 600°C, concluded that the mineral phase predominantly contributed to fluorescence. HARTLES and LEAVER (1952, 1955) suggested that fluorescence in teeth was, in part, due to thymidine or other pyrimidines accumulating in the dentinal tubules from nucleoprotein in odontoblastic processes. ARMSTRONG (1963) proposed a component, free of or bound to dentine protein, which could attain a fluorescent state independent of any protein u.v. absorption properties, since he was unable to explain dentine fluorescence in terms of u.v. absorption by aromatic amino acid residues.

There is neither certainty as to the nature of the primary unit of fluorescence nor a reasonable hypothesis of its relationship to health or disease in calcified tissues. Yet

* The opinions or assertions contained herein are those of the authors, and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

this luminescent property, as demonstrated in various proteins by UDENFRIEND (1962) and STEINER and EDELHOCH (1962), may be employed to follow modifications in molecular domain. If fluorescence in tooth and bone were more perfectly understood at the molecular level perhaps a better understanding of the process of dental caries and bone disease would be reached.

## MATERIALS AND METHODS

*Decalcification*

Normal human femur, dentine and enamel (1 g per 50 ml solution) were decalcified in dialysis cells (cellophane sheets, William Dixon, Inc., Newark, N.J.), using 10% disodium ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7·8. Decalcification was accomplished in 4 days at room temperature, with daily changes of EDTA solution. Insoluble material was washed with water and with ether, then desiccated. The dried material contained 4·3% calcium (oxalate method; SCOTT, 1939) and traces of phosphorus (FISKE and SUBBAROW, 1925).

*Peptide preparation*

Peptide mixtures were prepared by partial acid hydrolysis of EDTA decalcified tissue. For the hydrolysis, 1 g of protein from decalcified tissue per 12 ml of 3·6 *N* hydrochloric acid was maintained for seven days at 37°C in sealed tubes (SCHROEDER *et al.*, 1954). Acidic hydrolysate was adsorbed on Dowex 50W-X2 (hydrogen form), then poured into a column. Excess acid was removed by washing with water. The di- and tri-peptides were eluted with 4 *N* ammonium hydroxide. Eluates were concentrated under negative pressure at 40°C. The concentrated material was stored dry in a desiccator.

*Starch gel electrophoresis*

Starch gels (SMITHIES, 1955) were formed (12 g potato starch per 100 ml 0·75 *M* formic acid solution, pH 1·9) in trays 22 × 13 × 0·5 cm. Samples of peptide mixtures were solubilized and placed in the gel on 20 × 5 mm strips of Whatman No. 31 filter paper at the center of the slab. These strips held 0·07 ml in which 4·0 mg of peptide mixture was suspended. The 0·75 *M* formic acid solution was used as the electrolyte. Electrophoresis was at 400 V, 40 mA for 4 hr. After electrophoresis ultraviolet light (Mineralight No. SL-2537, 254 mμ) was directed on the gel surface to elucidate fluorescent zones.

*Cellulose column electrophoresis*

Larger amounts of separated peptides were obtained by column electrophoresis using cellulose-powder (Munktell's, Lot 474-476) as the supporting medium. A FLODIN-PORATH column (1954) was used in an U-tube arrangement. The column was 3 × 37 cm. The electrolyte system was 90% formic acid/acetic acid/water (27:120:853) at pH 1·9 (MICHL, 1959). Three hundred milligrams of the peptide mixture of bone or tooth protein were applied to the column, and the column was run at 600 V, 90 mA for 4 hr at 5°C. Elution with a fraction collector was time-regulated. Aliquots of

each fraction, after adjustment to pH 5, were analyzed by the colorimetric ninhydrin procedure of MOORE and STEIN (1948). The resultant peptide fractions were concentrated by lyophilization.

*Fluorescence measurements*

An Aminco-Bowman spectrophotofluorometer with 1P21 detector was employed for fluorescence characterization. Changes in pH affected neither fluorescent intensity nor excitation or emission maxima. The measurements were performed on solutions in $10^{-2}$ *N* NaOH. Relative values were derived for total fluorescence by the following relationship:

$$\frac{\text{area under emission curve (cm}^2\text{)}}{\text{sample concentration (mg/ml)}}$$

Areas were corrected for fluctuations in the *x*-axis of the recorder.

## RESULTS

Electrophoresis of total peptide mixtures on starch gel at pH 1·9 separated three distinct fluorescent zones. A photograph taken under u.v. light of a gel slab after electrophoresis of peptide mixtures is shown in Fig. 1. In bone, one diffuse cationic and two sharp anionic zones were revealed (Fig. 1a). Figure 1b presents the electrophoretic pattern of the isolate from a control Dowex column and identifies the anionic zones of the bone mixture as fluorescing constituents of the resin. Subsequent peptide mixtures had excess acid removed by repeated evaporation (Fig. 1c), thereby barring extraneous fluorescent substances.

Cellulose column electrophoresis of bone peptide mixture resolved twelve fractions (Fig. 2) as detected by ninhydrin reactivity. Major fluorescence (intensity per weight) was contained in zones 11 and 12. The elution pattern of dentine peptides was similar.

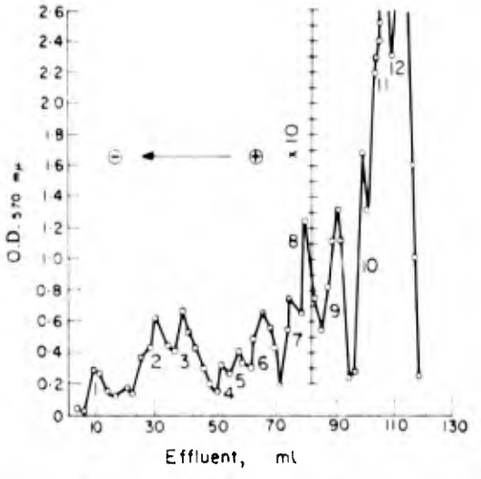


FIG. 2. Ninhydrin reactivity of bone peptides fractionated by cellulose column electrophoresis.

Ultraviolet absorption spectra of parent gelatins of bone, dentine and enamel are presented in Fig. 3 (The term "parent gelatin" refers to autoclaved EDTA insoluble decalcified protein. Although SCATCHARD (1944) originally used the term with soluble collagen, we feel that indication of a denatured product, rather than harshly treated commercial gelatin, is the prime connotation). Each have a broad maximum absorption band at about 280–285 mμ. The bone and dentine curves

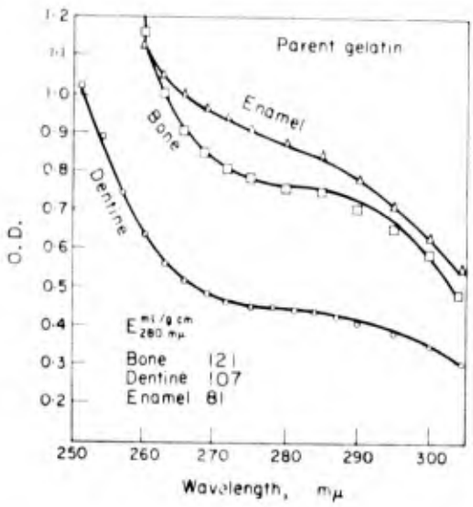


FIG. 3. Ultraviolet absorption spectra of parent gelatins of bone, dentine and enamel, in $10^{-2}$ *N* NaOH.

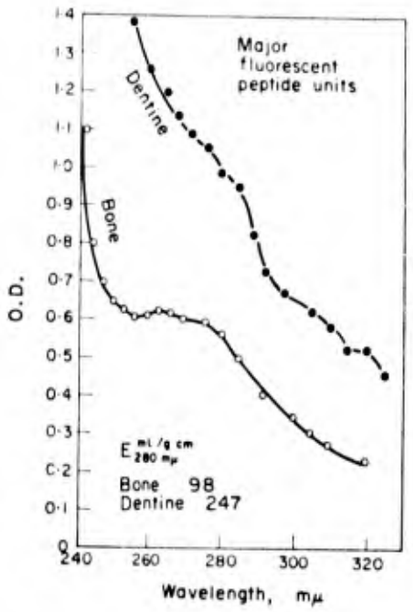


FIG. 4. Ultraviolet absorption spectra of the major fluorescing peptides of bone and dentine, in $10^{-2}$ *N* NaOH.

were similar in character. Absence of a minimum at about 275 m$\mu$ distinguished the enamel parent gelatin. Chromogen availabilities were indicated by relative extinction coefficients ($E^{\text{ml g}}_{280}{}^{\text{cm}}_{\text{m}\mu}$, based on weight) and were for bone, 121, dentine, 107, and for enamel, 81.

Figure 4 shows the u.v. absorption curves for the major peptides. The maxima were still around 280 m$\mu$ although in bone there was an additional band around 260 m$\mu$. Now, however, the extinction coefficient for dentine major peptide (247) exceeded that of bone (98).

TABLE 1. APPARENT EXCITATION AND EMISSION MAXIMA OF U.V. FLUORESCENT COMPONENTS OF HUMAN CALCIFIED PROTEIN (BONE, DENTINE AND ENAMEL)

| Component | Calcified protein: Bone λEX | Bone λEM | Dentine λEX | Dentine λEM | Enamel λEX | Enamel λEM |
|---|---|---|---|---|---|---|
| Parent gelatin | 323* | 400 | 315 | 400 | 325 | 402 |
| Peptide mixture | 321 | 406 | 321 | 403 | 292 | 408 |
| Major peptide fraction | 330 | 408 | 319 | 395 | — | — |

* m$\mu$ (uncorrected).

Fluorescence excitation and emission maxima are arranged in Table 1. The fluorospectral properties of parent gelatins of bone, dentine and enamel were, within instrument error, essentially identical: excitation 323, 315 325 m$\mu$; emission 400, 400, 402 m$\mu$, respectively. The peptide mixtures of bone and dentine remained unchanged with respect to fluorescent maxima. However, the excitation maximum of enamel pepide mixture shifted towards the blue (325–292 m$\mu$) and differed in this respect from its parent gelatin and from the other peptide mixtures. Due to insufficient starting material an enamel peptide mixture was not fractionated by cellulose column electrophoresis.

TABLE 2. TOTAL ULTRAVIOLET FLUORESCENCE* OF HUMAN CALCIFIED PROTEINS OBSERVED AT APPARENT EXCITATION MAXIMA

| Component | Calcified protein: Bone | Dentine | Enamel |
|---|---|---|---|
| Parent gelatin | 39·20† | 32·00 | 13·91 |
| Peptide mixture | 13·80 | 33·85 | 13·55 |
| Major peptide fraction | 23·00 | 93·90 | — |

* Determined by area under spectral curve. Corrected for $X$-axis variation.
† $Cm^2$/mg/ml.

Relative total fluorescence values are given in Table 2. In parent gelatins, bone fluorescence was greater than that of dentine, which fluoresced more than enamel (39·2, 32·0, 13·9 respectively). The total fluorescence of the bone peptides mixture diminished with respect to its parent gelatin (from 39·2 to 13·8), while the fluorescence

of dentine parent gelatin (32·0) and peptides mixture (33·85) did not differ appreciably. In each tissue the major peptide fraction as compared to its peptides mixture showed an increase in total fluorescence: bone 13·8–23, dentine 33·85–93·9 $cm^2/mg/ml$.

## DISCUSSION

Due to the high resolving power of starch gel supporting medium in electrophoresis, this method was chosen to detect fluorescent peptide fractions of calcified proteins. At pH 1·9 in an electric field all peptides should migrate as cations. No combination of amino acid residues would have an isoelectric point at a pH less than 1·9. This is especially so with the di- and tri-peptides which would exist in partial acidic hydrolysates of decalcified proteins. Consequently fluorescent zones on the anionic side of the origin (Fig. 1a) could not possibly be peptides. Investigation of the ultraviolet and infrared spectra, and chemical properties of these highly anionic fluorophores also indicated characteristics incompatible with those of peptides. Subsequently, by eluting Dowex free of protein peptides, these two zones proved to be fluorescing components of the resin (Fig. 1b). This was consistent with work which indicated the feasibility of obtaining practical industrial control of continuous ion exchange operations in certain processes by monitoring the fluorescence level of the resin (FLINT and EICHHOLZ, 1961). In those peptide mixtures which had excess acid removed by repeated evaporation the starch gel pattern did not include anionic zones (Fig. 1c).

Attention now focused on the major fluorescent fractions and the material from which these fractions were derived. The ultraviolet absorption spectra showed differences among the various tissues, which indicated that enamel protein structure was unlike dentine and bone protein. This observation was based solely on the absence of a minimum around 275 $m\mu$ in mature enamel protein. Although the u.v. absorption curves shown here for decalcified proteins lack narrow maxima and minima, the absence of any suggestion of a minimum in enamel protein appeared to distinguish this tissue. Enamel parent gelatin had the lowest quantity of u.v. absorbing material, as evidenced by extinction coefficients. This was consistent with bone and dentine having about 90 per cent collagen in the organic matrix and enamel about 60 per cent protein in the organic matrix.

The major peptide fractions essentially retained the character of the parent gelatin absorption curves. A new band appeared in bone at about 262 $m\mu$. (Simple pyrimidines absorb in this region.) The extinction coefficient for major peptide fraction of dentine exceeded that for bone by about two and a half times, while, in the parent gelatin solutions, the extinction coefficient for bone slightly exceeded that of dentine. There was also a difference in the relationship of parent gelatin to major peptide fraction. The $E$ of bone parent gelatin was higher than that of the major fluorescing peptides of bone. Conversely, the $E$ for major peptides of dentine was higher than for dentine parent gelatin. This indicated that the major fluorescing peptides of bone, those in zone 12, have lost a considerable quantity of the chromogen which absorbs at 280 $m\mu$.

The work of EASTOE (1955) shows that there are close similarities in the amino acid composition of collagen and gelatin. Thus it was valid to use published amino acid data of collagen to compare findings in our parent gelatins. Our preparation of collagen was such that free amino acids were not retained, and gelatinization should not have destroyed any amino acid residue. Assays of the materials studied here (EASTOE, 1955; PIEZ, 1961; HESS, LEE and NEIDIG, 1953) report 16–45 phenylalanine residues and 6–9 residues of tyrosine per 1000 residues. Tryptophan values for bone and dentine are not given; this amino acid is largely destroyed in acid hydrolysis and no attempt was made to determine it separately. HESS *et al.* employing microbiological methods found 0·12 per cent tryptophan in mature enamel protein. Phenylalanine may be eliminated as a contributing chromophoric group on the basis of its relatively insignificant molar extinction (phenylalanine, 150; tyrosine, 1520; tryptophan, 6000). If tyrosine were assumed to be the sole chromogen of the parent gelatin solutions, the calculated relative extinction values would be consistent with the molar extinction coefficient of this amino acid, and the fact that tyrosine constitutes 0·6–0·9 per cent of decalcified protein. Thus, the amino acid could account for nearly all the 280 m$\mu$ absorption in bone, dentine and enamel parent gelatin. This apparently was not the case in the major fluorescing peptide of bone, since the value of $E$ was diminished (Fig. 4).

The aromatic amino acids contribute to fluorescence as well as absorptivity in proteins. In order to observe the greatest fluorescent yields possible, the protein chain was opened by heat denaturation, thus exposing the aromatic groups. Under these conditions, phenylalanine fluorescence is not observed in the presence of other aromatic amino acids (TEALE, 1960). Fluorescence is detected in protein of low tyrosine content, but only in the absence of tryptophan. So, presuming an absence of tryptophan in bone and dentine protein, tyrosine could conceivably account for nearly all the protein fluorescence. However, in proteins containing tryptophan the fluorescence spectrum of this residue alone is observed, even to the exclusion of tyrosine when present.

That the fluorescent characteristics of the parent gelatins were essentially identical coincides with the similarity of their u.v. spectra. The uniqueness of the enamel parent gelatin absorption (no minimum at 275 m$\mu$) was not evident in the fluorescent excitation and emission maxima since these maxima in all three tissues were the same. However, enamel showed distinction in the peptides mixture with the shift to shorter fluorescent (292 m$\mu$) wavelengths. The fluorescent maxima for bone and dentine indicated that the main fluorogens of bone and dentine were separated.

The percent decalcified protein content in bone, dentine and enamel organic matrix agreed with their observed total fluorescence, and extinction values. This relationship between these calcified tissues may be a matter of less of the same material in enamel fluorescing at 400 m$\mu$ than in bone and dentine. $E$ values of the three tissues coincide with this explanation. The presence of structural differences must be considered, too, based on the absence of a minimum at 275 m$\mu$ in the u.v. absorption spectrum of enamel protein, and the shift in excitation maximum to 292 m$\mu$ in its peptide mixture.

In the total fluorescence of peptide mixtures, that of the bone mixture diminished by about one-half. The question of an inner filter effect arises. Since the solutions employed here were of concentrations such that they fell on the straight line section of the fluorescent-intensity-versus-concentration curve one could discount such an effect in diminished fluorescent efficiency. As well, the use of right angle detection assured optimum response as a biologic curve. This decrease in total fluorescence from parent gelatin to peptide mixture is real, and would tend to coincide with the proposed loss of chromogen evidenced in the u.v. absorption findings. The isolation of both the major peptide fractions of bone and dentine from the peptide mixtures was expected to show an increase in total fluorescence and did. Since the values are on a weight basis, removal of components of low "fluorescence efficiency" would elevate the total isolated peptide fluorescence value.

Two facts continued to press the question of whether tryptophan was present: (1) tyrosine could not totally account for u.v. absorption, and (2) if tryptophan were present, there should be an absorbed energy transfer from tyrosine to tryptophan with respect to total fluorescent intensity. By use of modified fluorometric procedures, tryptophan has been found in bone (HOLMAN and MANCEWICZ, 1964) at about 0·3 g per 100 g decalcified protein and dentine at 0·17 g per 100 g. Identification was in basic hydrolysates of pH 6·0; $\lambda_{ex}$ 280 m$\mu$, $\lambda_{em}$ 355 m$\mu$, the maxima for tryptophan. Comparable values for tyrosine were $\lambda_{ex}$ 275 m$\mu$, $\lambda_{em}$ 316 m$\mu$. Tyrosine was also noted. In these basic hydrolysates there was no response to specific excitation at 320 m$\mu$. A rough correction of these wavelength values, using the factors of WHITE (1960), brought the excitation maxima to the ideal of being identical with the u.v. absorption for tyrosine and tryptophan. Corrections, however, did not reduce the 400 m$\mu$ emission of our preparations in the present study to the 350–360 m$\mu$ emission region of indole derivatives (COWGILL, 1963).

In bone with 0·86 g tyrosine and approximately 0·3 g tryptophan per 100 g, the tryptophan fluorescence would dominate. For example, in human serum albumin, with a ratio of 17:1, tryptophan entirely masks the fluorescence of tyrosine. In our preparations the tryptophan emission region (350–360 m$\mu$) was masked in the parent gelatins by strong fluorescence in the 400 m$\mu$ area. This fluorescence persisted in peptide mixtures and in the major fluorescing fraction. Apart from this unidentified fluorescent component there is evidence that tyrosine and tryptophan contribute to the material of parent gelatin which absorbs at 280 m$\mu$. These two amino acids as well are partially responsible for the fluorescence of the preparations studied here.

Consequently, the total fluorescence of calcified proteins occurs as a combination of excitation of three or more fluorescing molecules, tyrosine and tryptophan to the extent of their absolute concentration and another fluorophoric group(s) presently unidentified.

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**Résumé**—Des peptides sont préparés par hydrolyse acide partielle de protéine insoluble de dent et d'os. L'électrophorèse à colonne de cellulose est utilisée pour séparer les principaux peptides fluorescents. A partir de gélatines "parents", de mélanges peptidiques, et de fractions peptidiques principales des protéines calcifiées, les mesures suivantes sont réalisées: absorption u.v. et excitation fluorescente et spectre d'émission, coefficients d'extinction et fluorescence totale. Il semble que le tryptophane et la tyrosine participent à la constitution de gélatine "parent", qui absorbe à 280 mμ; ils semblent partiellement responsables de la fluorescence des préparations étudiées. La fluorescence totale des protéines calcifiées semble résulter d'une combinaison de l'excitation de 3 molécules fluorescentes au moins, comportant la tyrosine et le tryptophane, dans la mesure de leur concentration absolue, et un ou plusieurs groupes actuellement non identifiés.

**Zusammenfassung**—Durch partielle Saurehydrolyse unloslichen Eiweisses von Zähnen und Knochen wurden Peptide gewonnen. Um die grösseren, fluoreszierenden Peptide zu trennen, wurde die Zellulose-Saulenelektrophorese angewandt. Folgende Messungen wurden unter Verwendung von Knochen—bzw. Dentingelatinen, Peptid-Mischungen und Fraktionen der grösseren Peptide von verkalkten Proteinen angestellt: UV-Absorption, Fluoreszenzerregung und—emissionsspektren, Extinktionskoeffizienten und totale Fluoreszenz. Es gibt deutliche Hinweise dafür, dass Tryptophan und Tyrosin am Aufbau des Knochen—und Dentingelatins, welches bei 280 mμ absorbiert, beteiligt sind, und dass sie zum Teil für die Fluoreszenz der hier untersuchten Zubereitungen verantwortlich sind. Die gesamte Fluoreszenz verkalkter Proteine kommt als eine Kombination der Erregung von drei oder mehr fluoreszierenden Molekülen zustande. Hierbei sind Tyrosin und Tryptophan bis zum Ausmass ihrer absoluten Konzentration sowie eine oder mehrere andere fluorophore, derzeit noch unerkannte Gruppen beteiligt.

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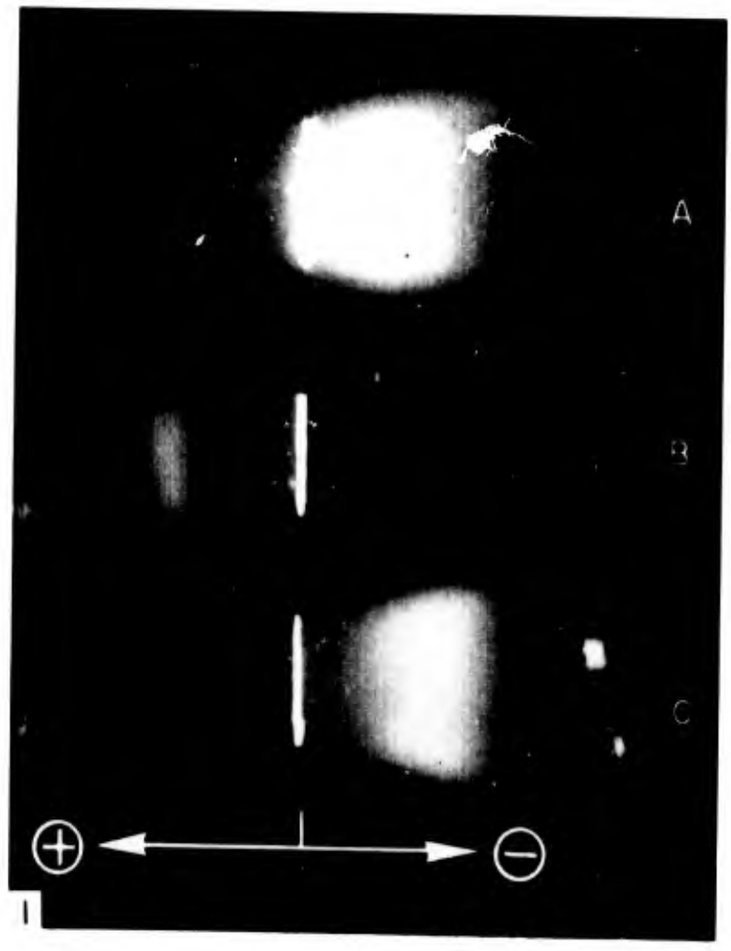


FIG. 1. A starch gel slab photographed under ultraviolet light showing the electrophoretic separation of fluorescent components of Dowex resins from the fluorescent component of bone and dentine acid hydrolyzates: A. Bone peptides, prepared on Dowex; B. Dowex components; C. Dentine peptides prepared without Dowex.