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The Fluorescence of Teeth under Ultraviolet Irradiation

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The observation that teeth fluoresce when irradiated with ultraviolet light was reported by Stubel (1911). He studied a whole series of tissues and found that the teeth exhibited an intense blue fluorescence second only in brilliance to the lens of the eye. Some years later, Benedict (1928) noted that dentine fluoresced much more strongly than enamel and with a seemingly bluer light. He observed that the white spot indicative of 'beginning' dental caries did not fluoresce. He produced a similar effect by scratching through a paraffin-coated tooth and placing in dilute acetic acid overnight. Benedict also noted that ashed enamel did not fluoresce, nor did dentine which had been boiled in 50 % sodium hydroxide, whilst on decalcifying dentine with dilute nitric acid the remaining organic matrix retained its fluorescence to an appreciable extent. Tiede & Chomse (1934) were of the opinion that it was the organic protein compounds of the teeth which were responsible for the phenomenon. They said that teeth extracted with alkaline glycerol retained enough organic matter to fluoresce, but that the property was lost after treatment with hydrogen peroxide and ammonia. Glasser & Fonda (1938), on the other hand, believed that the fluorescence was linked with the mineral phase. They used ox teeth, presumably the enamel only, since they stated that their material contained 1 % protein. According to these workers the fluorescence was destroyed by burning out the organic content at 600° but was increased by heating at 400°. They found that after 'solution of the teeth in acid and precipitation with alkali' the fluorescence was restored by heating at 400°. Bodecker (1939) put forward the suggestion that dental lymph was responsible for the fluorescence, although vital and non-vital teeth both fluoresce. Hadjioloff & Russewa (1943) observed that enamel, dentine and cementum appeared blue under ultraviolet light and that enamel and dentine sometimes appeared yellow. They reported that by decalcifying with a half-saturated solution of sodium chloride in dilute hydrochloric acid the teeth lost their ability to fluoresce.

The phenomenon of tooth fluorescence poses two main questions. First, what substance or substances are responsible and, secondly, why does the carious lesion cease to fluoresce? The answer to these two questions may be of fundamental importance in the aetiology of dental caries. If, for example, the active material should prove to be organic in nature, it would mean that disintegration of organic matter occurs at a very early stage in the carious process. The object of the present investigation was to observe the fluorescence in various tooth specimens and to explore techniques for extracting the active substances from the teeth, with a view to finding some information concerning their chemical nature.

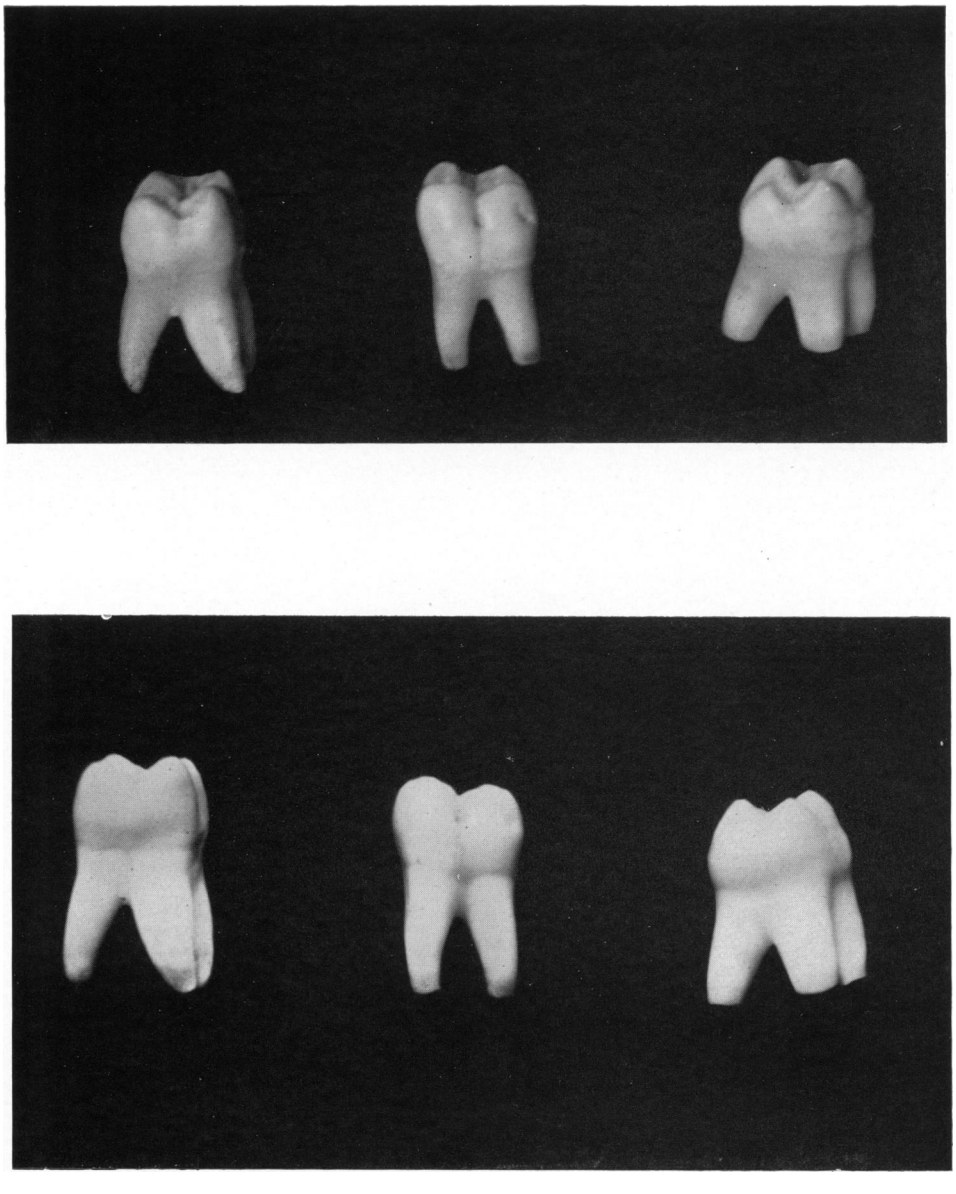
EXPERIMENTAL

Observations on the fluorescence of teeth

A 'dark bulb' mercury-vapour lamp was used routinely as a source of ultraviolet radiation. The appearance of the fluorescence was compared with that produced by a 'Hanovia' fluorescence lamp, Model 11, with Wood's glass filter which transmits in the region of 366 mμ. No differences were detected. Prior to their examination, teeth were freed from adhering tissues, calculus and stains by careful use of brush and pumice followed by a thorough washing in water. Observations were made on whole teeth and on sections.

Sound teeth

(i) *Enamel.* Enamel normally fluoresced bluish white but occasionally tinged with yellow. Pl. 2 shows a group of three sound teeth photographed under artificial white light and under ultraviolet light; the differences in colour intensity are lost in the black and white print, but an impression can be gained of the lustre of the fluorescent light.



A group of three teeth photographed in artificial white light (above) and in ultraviolet light (below).

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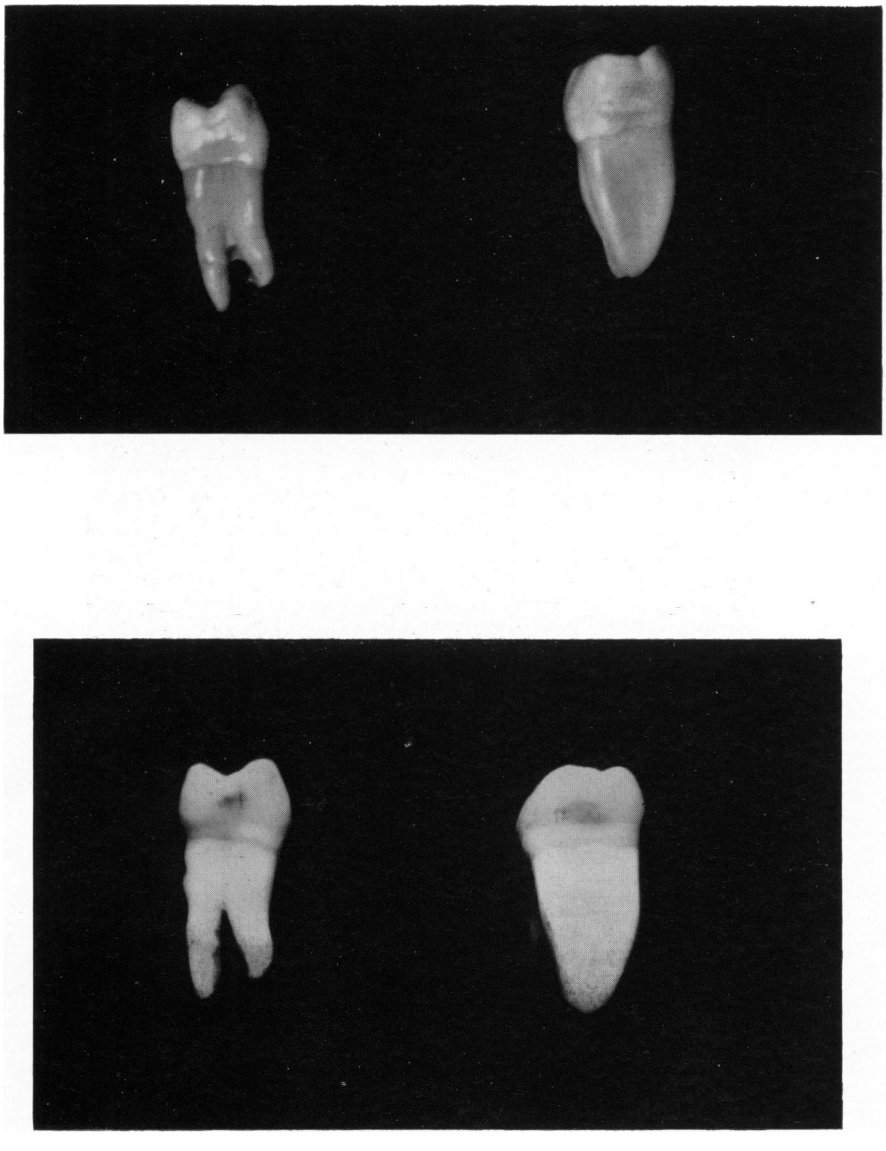
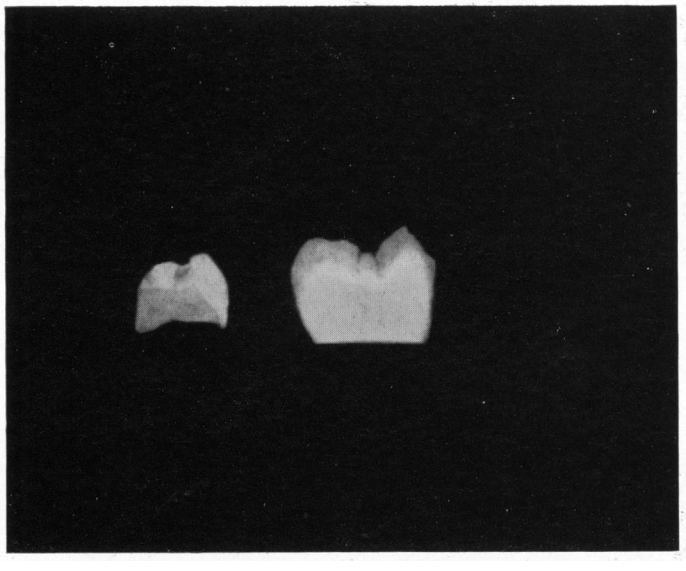


Early carious lesions showing as whitish patches when photographed in artificial white light (above), and as black non-fluorescent areas when photographed in ultraviolet light (below).



Photograph of two tooth sections in ultraviolet light. That on the left has been refluxed with ethylene glycol containing 3% potassium hydroxide, the intensity of the dentine fluorescence is markedly reduced compared with the normal section on the right.

(ii) *Dentine.* Dentine fluoresced much more strongly than did the enamel with an intense blue-violet light. The colour was most intense in the apical regions, particularly in some immature teeth with open apices.

(iii) *Cementum.* The appearance of the fluorescence was similar to that of dentine but the colour was less intense.

Carious teeth

(i) *Carious enamel.* This did not fluoresce, the carious areas appearing black. In the early lesions the whitish patches had lost their fluorescence (Pl. 3). In section, the loss of fluorescence extended with the lesion.

(ii) *Carious dentine.* The blue-violet colour was absent and the surfaces of the lesion appeared black or dark brown. When the carious dentine was carefully ground away an orange-brown fluorescence was observed, which at the base of the lesion, after removal of practically all the carious material, became pinkish orange in colour.

(iii) *Calculus.* Patches of calculus on the teeth could be distinguished readily as they fluoresced with a reddish orange colour.

The effect on tooth fluorescence of etching with dilute acid

We repeated Benedict's (1928) experiment of coating a tooth completely with paraffin wax, scratching through the coat to the enamel and immersing in 2N-acetic acid overnight. The tooth was then removed, washed and the wax scraped away. The scratch appeared as a black line when the tooth was viewed in ultraviolet light. The same result was obtained with 2N-HCl. If a drop of the dilute acid was placed carefully on the smooth surface of enamel, or if the tip of the enamel-coated crown was dipped into the acid and left overnight in a closed vessel no effect was noticed on the fluorescence. We are of the opinion that the dark appearance of the waxed etched tooth is a combination of optical factors and the forcing into the scratch of wax during the scraping process. A similar effect can be produced by scratching a waxed tooth with a fine glass cutter and then scraping off the wax.

The fluorescence of dentine

Dentine contains much more organic material and fluoresced much more strongly than the enamel. It is probable therefore that the fluorescing substances in dentine are associated with the organic fraction.

Ethylene glycol extraction of dentine. A longitudinal section through the crown of a tooth was placed in ethylene glycol containing 3% (w/v) KOH, a process used for extracting organic matter from dentine (Crowell, Hodge & Line, 1934), and refluxed for 90 min. The section was then removed, washed thoroughly with water until the washings were no longer alkaline to phenolphthalein and dried. On examining under ultraviolet light the fluorescence of the enamel was little changed in appearance but that of the dentine was very much weaker and less blue in colour (Pl. 4). The alkaline ethylene glycol fluoresced with a strong blue colour. It appeared therefore that this treatment, in removing the organic fraction of the dentine, had also extracted some fluorescing substances. It was decided therefore to extract a larger amount of dentine.

The crowns were removed from a number of teeth and the roots stripped of adventitious material. Any caries, calculus and stains were removed with a bur, brush and pumice followed by washing in water. The contents of the root canal were removed with a dental bur. About 100 g. of the roots so prepared were split into fragments and ground in a percussion mortar until they passed through a 30-mesh sieve. Analysis of samples of this powdered dentine (plus a little cementum) showed that it contained 3·5% N (Kjeldahl). The dentine (100 g.) was then refluxed for 3 hr. with 250 ml. of ethylene glycol containing 3% (w/v) KOH. It was necessary to stir the mixture mechanically during the heating to avoid bumping and uneven heating. This process was in effect hydrolysis by dilute alkali at a high temperature (180–200°). After refluxing, the mixture was dark amber in colour but the liquid phase exhibited strong fluorescence in the meniscus or when diluted with water. The supernatant liquid was decanted and the residue washed about ten times with 50 ml. portions of boiling water until the washings were no longer pigmented and fluoresced only weakly. Samples of the extracted dentine now contained less than 0·05% N. The ethylene glycol extract plus the washings were centrifuged and filtered through an asbestos pad to remove suspended particles. The resultant solution was clear, of a deep-amber colour and fluoresced strongly at the surface. A drop of the solution placed on a filter paper produced an intensely blue fluorescing spot. Nitrogen determinations on the solution showed that it contained approximately 66% of the N originally present in the dentine.

This leaves more than 30% of the N unaccounted for. A strong ammoniacal and 'amine' smell was noticed during the digestion. In order to investigate these volatile compounds and to try and obtain a nitrogen balance sheet, a small quantity of dentine (1·7 g.) was boiled gently in alkaline ethylene glycol for 90 min.; the volatile compounds evolved were led through an excess of dilute H_2SO_4 to trap any basic material. The resulting solution was evaporated and was found to contain 15% of the N originally present in the dentine. The glycol extract contained 73% and the washed residues 2·6% of the total N. This heating was rather more gentle than the previous vigorous refluxing and the dentine was less thoroughly extracted; even so, only 90% of total N was recovered. We cannot yet account for the missing 10% except to suppose that it is evolved as volatile compounds not absorbed by dilute H_2SO_4.

Treatment of ethylene glycol extract. The diluted ethylene glycol extract was acidified with HCl to pH 1, shaken at room temperature with 10 g. of activated charcoal (British Drug Houses Ltd.) and filtered. This treatment removed most of the pigment and the fluorescence of the solution was markedly diminished. Two further treatments with charcoal rendered the filtrate colourless and almost non-fluorescent. The fluorescing materials and the pigments in the extract had therefore been adsorbed on the charcoal. The charcoal was washed thoroughly with successive quantities of cold water until the washings were non-fluorescent. Very little fluorescent material was removed by this treatment.

Elution from charcoal. Repeated extraction with 92% (v/v) ethanol produced a pale-yellow solution fluorescing blue, but the active materials were only removed slowly. The most satisfactory eluant for recovering the adsorbed

substances was a solution containing 5 vol. ethanol, 4 vol. water and 1 vol. of ammonia (sp.gr. 0·88). This produced a deep-amber-coloured eluate which fluoresced strongly. Unfortunately, some pigmented impurities already in the charcoal were also eluted by this treatment, but most of them could be removed later as they were relatively insoluble in absolute ethanol.

Treatment of eluate. The combined ammoniacal eluates were taken to dryness under reduced pressure at 50–60°, yielding a yellow amorphous solid (2·5 g.) containing 8·2% N (0·55% N, referred to 100 g. of original dentine). Thus about 15% of the total dentine nitrogen is contained in the eluate. The amorphous solid was dissolved in 50 ml. absolute ethanol and termed solution *A*. (A small amount of material, mainly impurities from the charcoal, did not dissolve.) The solid was also soluble in water and in methanol, partially soluble in acetone, but insoluble in ether, chloroform or light petroleum.

Paper chromatography. Chromatograms using both butanol/acetic acid and propanol/ammonia systems showed the presence of ninhydrin-positive material (i.e. amino-acids or peptides) but resolution was poor. The fluorescing substances tended to 'streak' the whole length of the chromatogram with areas of concentration not necessarily associated with ninhydrin-positive material.

A portion of solution *A* was again taken to dryness and the solids hydrolysed by refluxing with 6N-HCl for 8 hr. The hydrolysate (strongly fluorescent) was taken to dryness and extracted with successive small amounts of absolute ethanol, until finally only a small non-fluorescing ethanol-insoluble residue remained. The combined extracts (solution *B*) were centrifuged to remove small particles of suspended matter. Paper chromatograms of this solution showed the presence of several amino-acids normally present in dentine which were identified by comparison with chromatograms of known amino-acids running adjacently. The fluorescing materials were still not resolved although at high concentration there was a tendency to form crescents at a fairly constant R_F value. There was no correlation between the position of the amino-acids and the fluorescence.

Adsorption chromatography. Solution *B*, diluted with ethanol, was passed through a column of activated alumina (Peter Spence and Co. Ltd.). On developing with absolute ethanol a dark-brown band stayed at the top of the column but much fluorescent material passed through. Development was continued until the eluate was almost non-fluorescent. The eluate, when concentrated, was bright yellow and fluoresced strongly with a bluish colour. This eluate was taken to dryness and yielded a yellowish gum soluble in water, methanol and ethanol but insoluble in ether, chloroform and light petroleum. The gum was redissolved in methanol and to this solution was added an ethereal solution of diazomethane (prepared from nitrosomethylurea) and left to stand overnight. On evaporating to dryness the residue was almost completely soluble in $CHCl_3$ and still fluoresced strongly. The yellow $CHCl_3$ solution was now passed through an alumina column, whereby four distinct fluorescent zones developed and each was collected separately as it was washed off the column. When no further fluorescent material could be obtained by washing with $CHCl_3$, development was continued with ethanol. By this means three or four rather ill-defined fractions were obtained. All of the fractions were yellow in colour, some strongly, others only slightly pigmented.

Ultraviolet-absorption spectra. Each fraction was taken to dryness, yielding a very small quantity of gum, redissolved in absolute ethanol and the absorption spectra determined using a Beckman spectrophotometer. All fractions showed much end absorption but inflexions were observed at 270 mμ. or 256 mμ.

Extraction of dentine with butanolic potassium hydroxide

In order to minimize contamination of the materials with impurities from the charcoal it was decided to omit the adsorption stage and use a more volatile extracting solvent which could be removed by distillation. The solvent chosen was *n*-butanol. Dentine (60 g.) was refluxed for 6 hr. with *n*-butanol containing 5% KOH. This treatment did not remove the organic matter as completely as did alkaline ethylene glycol; the residue after extraction still contained about 0·3% N. The supernatant liquid was separated from the dentine residue, neutralized and taken to dryness on a boiling-water bath under reduced pressure. The residue was extracted with methanol and insoluble non-fluorescent material removed by centrifuging. The methanol solution was again taken to dryness and extracted with 92% (v/v) ethanol; this gave a deep-yellow solution strongly fluorescing blue, the solids of which contained 9·8% N.

Adsorption chromatography. The fluorescent ethanolic solution was poured on to a column of activated alumina and the chromatogram developed as follows: (*a*) *Development with 92% ethanol.* Fraction *A* ran straight through the column and did not appear as a band. It was fractionated on another column into two components A_1 and A_2 by developing with absolute ethanol and 50% (v/v) aqueous ethanol. Fraction *B*, a small distinct fluorescent band. Fraction *C*, a long straggling fluorescent fraction. (*b*) *Development with 10% (v/v) ammonia (sp.gr. 0·88) in ethanol.* Fraction *D*, a distinct band which appeared to develop from a clear stationary yellow band. (*c*) *Development with 25% (v/v) ammonia in ethanol.* Fraction *E*, a large fraction appearing as a yellow band on the column. (*d*) *Development with water.* A large fraction was obtained, brown in colour, rather arbitrarily divided into fractions *F* and *G*.

Each of these fractions (*A–G*) was taken to dryness and redissolved in absolute ethanol. In all cases, particularly so in *E–G*, white, alcohol-insoluble, non-fluorescent solids were removed by this treatment. These solids were ninhydrin-positive.

Acetylation of fractions. Each fraction was again taken to dryness and taken up in a few ml. of pyridine, insoluble non-fluorescent matter being filtered off. An equal volume of acetic anhydride was added and the solutions left for 24 hr. Each solution was taken to dryness several times from absolute ethanol to remove the last traces of pyridine and acetic anhydride. Each fraction was now dissolved in $CHCl_3$ and filtered, where necessary, from the very small insoluble residue.

These $CHCl_3$ solutions of the acetylated fractions were then used for chromatography on activated alumina. After acetylation, fractions A_1, A_2, *B*, etc., were named A_1X, A_2X, *BX*, etc. Each fraction was adsorbed on an alumina column, developed first with $CHCl_3$ then with ethanol and, if necessary, with dilute aqueous ammonia. In all, some forty-eight individual fractions were obtained and the ultraviolet-absorption spectrum of each was determined. Many of the fractions exhibited end absorption only which will not be described further.

Fractions were numbered serially, e.g. fraction EX_4 is the fourth fraction obtained when the column containing EX was developed, EX_{4A} is the first fraction when EX_4 was rechromatographed. All the fractions obtained fluoresced and most of them were coloured yellow. Only those which exhibited marked selective absorption in the ultraviolet are described below:

A_1X_6. A very small fraction removed by developing with water; $\lambda_{max.}$ 272 mμ., $\lambda_{min.}$ 230 mμ.

A_2X_1. A small fraction running straight through in $CHCl_3$; $\lambda_{max.}$ 258 mμ., $\lambda_{min.}$ 234 mμ.

A_2X_3. A distinct yellow band developed with $CHCl_3$; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 232 mμ.

A_2X_4. A small fraction developed with ammonia; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ below 220 mμ.

CX_4. A yellow ethanol-developed fraction; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 230 mμ.

EX_{5A}. A small pale-yellow fraction running straight through in $CHCl_3$; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 233 mμ.

EX_{5O}. Ethanol fraction; $\lambda_{max.}$ 265 mμ., $\lambda_{min.}$ 230 mμ.

FX_3. A 'tailing' fraction developed with $CHCl_3$; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 230 mμ.

FX_{5B}. A small fraction developed with ethanol; $\lambda_{max.}$ 265 mμ., $\lambda_{min.}$ 238 mμ.

GX_3. A small fraction developed with $CHCl_3$; $\lambda_{max.}$ 268 mμ., $\lambda_{min.}$ 235 mμ.

GX_{4A}. A small fraction running through in $CHCl_3$; $\lambda_{max.}$ 258 mμ., $\lambda_{min.}$ 227 mμ.

GX_{5A}. A small fraction running through in $CHCl_3$; $\lambda_{max.}$ 270 mμ., $\lambda_{min.}$ 230 mμ.

The remaining fractions either had end absorption only or slight inflexions in the region of 255 or 270 mμ.

Since the amount of material present in each fraction was so small we were not at this stage able to determine even rough $E^{1\%}_{1\,cm.}$ values.

Absorption between 300 *and* 450 *mμ.* Since the radiation exciting the fluorescence is in the region of 366 mμ., absorption must be occurring near this wavelength. Concentrations which showed selective absorption at 256 or 270 mμ. showed only continuous absorption at longer wavelengths. The fractions were concentrated as much as possible, and the region 300–450 mμ. carefully examined for selective absorption but without success. All fractions showed considerable continuous absorption in this region. We can only conclude that the absorption of the active material is completely masked by the presence in our extracts of relatively vast amounts of impurities.

Tests on the fluorescent extract of dentine. The neutralized butanolic KOH digest of dentine was taken to dryness, extracted with absolute ethanol and the non-fluorescing residue discarded. This orange-yellow solution was evaporated, the residue dissolved in water and subjected to the following tests. When sodium dithionite ($Na_2S_2O_4$) was added the fluorescence, but not the pigment, disappeared; it was regenerated on shaking in air or by adding a few drops of H_2O_2 solution. Acid $KMnO_4$ or bromine water destroyed the fluorescence irreversibly. The addition of H_2O_2 did not appear to affect the fluorescence. (It should be mentioned that freshly voided human urine fluoresced moderately strongly with a blue light when irradiated. The fluorescence behaved in exactly the same way, when treated with the above reagents, as did the dentine extract.)

Acetylation of the dentine extract. Treatment of the extract (after removing the solvent) with pyridine and acetic anhydride rendered it partially soluble in ether. This ethereal solution was bright yellow in colour and fluoresced blue-green. It was separated by chromatography on alumina into a major and two minor components. The major fraction fluoresced blue in ether but on evaporating and redissolving in water the fluorescence was bright yellow. This fluorescence was destroyed by sodium dithionite and the pigment changed from yellow to orange; shaking in air restored the fluorescence and the yellow colour. The yellow fluorescence was very similar to that of riboflavin, but when the latter was treated with pyridine and acetic anhydride, evaporated and extracted with ether, the resulting solution still fluoresced bright yellow.

The resistance of the fluorescing substances to acid digestion. When carrying out N determinations by the Kjeldahl method on the original extracts it was noted that after digestion with H_2SO_4 the blue fluorescence was not completely destroyed.

Acid hydrolysis of the alkaline ethylene glycol extract or the butanolic extract with 6 N-HCl for 8 hr. did not destroy the fluorescence. With 6 N-HCl in a sealed tube at 100°, the fluorescence was destroyed completely.

Fluorescent material remaining after sulphuric acid digestion. After digestion with conc. H_2SO_4, the digest was diluted with water and neutralized with excess barium carbonate, the mixture being stirred vigorously for 30 min. and then filtered. The filtrate was taken to dryness and extracted with absolute ethanol. This ethanolic solution fluoresced with a blue-violet colour; the absorption spectra had $\lambda_{max.}$ 256 mμ., $\lambda_{min.}$ 228 mμ.; $E^{1\%}_{1\,cm.}$ on our crude material was 138.

It is possible that, during the digestion, condensation reactions have occurred resulting in the formation of fluorescent compounds not present in the original teeth. Nevertheless, it is of interest to pursue this aspect and to try and determine the nature of the reactants.

Extraction of powdered dentine with diethyl ether. About 20 g. of powdered dentine were dried at 100° and continuously extracted with ether for 6 hr. The resulting solution of dentine lipids had only a very faint fluorescence, and the fluorescence of the dentine powder appeared unchanged.

DISCUSSION

Our experiments so far are of an exploratory nature and the results descriptive. We have shown that alkaline ethylene glycol and alkaline *n*-butanol digests of powdered dentine contain fluorescent substances. The dentine residue after such treatment has lost much but not all of its fluorescence. We are satisfied that at least some of these substances are organic in nature since they can be acetylated and methylated and rendered soluble in chloroform. It would also appear that fluorescence is not directly associated with the amino-acids of dentine. Some fluorescing material is present after prolonged heating with concentrated sulphuric acid. Our extracts contained many fluorescent fractions, but these may well be fission products of the active material existing in the dentine.

Blue fluorescent substances occur in many tissues, notably the skeleton, the eye, skin, hair, nails and in the urine. Pirie & Simpson (1946) isolated a substance showing blue fluorescence from the

choroid of the dogfish eye; chemical and spectroscopic evidence make it probable that this substance is xanthopterin. Pterins have also been shown to occur in urine, and uropterin has been proved to be identical with xanthopterin (Koschara, 1943). Although many of our extracts were yellow in colour we have no evidence to suggest that they contain xanthopterin. The ultraviolet-absorption spectrum of this pterin in neutral solution has maxima at 275 and 375 mμ. (Totter, 1944). Some of our fractions had a maximum in the region of 270 mμ., but on no occasion so far has absorption at 375 mμ. been encountered. Goodwin & Srisukh (1950) isolated a yellow pigment from locust eyes which they identified as a pterin closely resembling xanthopterin. This compound absorbed selectively in the region 270–280 mμ. with only a slight band at longer wavelength.

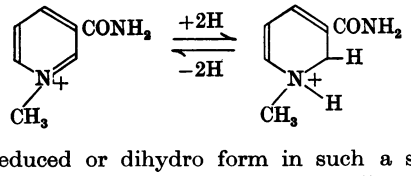
The information we can obtain from spectroscopic examination of our very impure fractions is scanty, and in view of the findings of Goodwin & Srisukh (1950) it is possible that a peak of low intensity is hidden by much irrelevant absorption. Even so, the intensity of the 375 mμ. band of xanthopterin is 0·27 of that of the 270 mμ. band, and we feel that with a ratio of this order we should have observed absorption at the longer wavelength if the 270 mμ. band of our extracts was indeed a component of a pterin spectrum.

Also the fact that the florescence shown by our extracts is unstable to sodium dithionite and potassium permanganate suggests that they do not contain xanthopterin. Becker & Schöpf (1936) report that the fluorescence of pterins is unaffected by these two reagents. Our fractions having selective absorption at 270 mμ. were obtained from the alumina chromatogram at widely separated intervals; most of our fractions had strong end absorption with no selective maxima. It is possible that the substance responsible for the selective absorption may have little to do with the fluorescence. Our activating ultraviolet light was in the region of 366 mμ.; this means that the fluorophoric group must be absorbing in this region. The fact that so far we have not found any selective absorption between 350 and 450 mμ. suggests that the continuously absorbing impurities in our extracts are present in such quantity as to mask completely any selective absorption. The selective absorption at 270 mμ. cannot by itself be responsible for the fluorescence since our light source does not contain radiation at this wavelength. It may be, of course, that the 270 mμ. peak is one component of a spectrum, the longer wavelength of which is hidden by continuously absorbing impurities. This must await further investigation.

One of our acetylated fractions fluoresced bright yellow in aqueous solution, appearing very similar to a solution of riboflavin. It was sensitive to dithionite, but unlike riboflavin the visible pigment did not disappear but changed to orange. In ethereal solution our fraction fluoresces blue; if riboflavin is subjected to acetylation procedures, the ethereal solution still fluoresces yellow. We can only conclude that riboflavin is not responsible for the yellow fluorescence in our extract.

The observation that some fluorescence persists after digestion with concentrated sulphuric acid is of interest, although we realize that it may be due to artifacts. Certain compounds containing nitrogen in the ring, e.g. pyrimidines, are known to be broken down with difficulty by this process. Ring compounds of the pyrimidine series which lack ethylene linkages within the ring may exhibit end absorption but lack any marked degree of selective absorption (Heyroth & Loofbourow, 1934). Pyrimidines may arise in our extracts from the breakdown of nucleoprotein. O'Brien (1944) has reviewed the literature concerning the occurrence in urine of a substance which yields a blue fluorescing compound (F_2) described by Najjar & Wood (1940). Its excretion is increased in proportion to the nicotinic acid intake; it has now been isolated and has a greenish blue fluorescence in alkaline and a blue fluorescence in acid solution (Huff & Perlzweig, 1943; Ellinger & Coulson, 1943; Najjar, White & Scott, 1944). It would appear to be a carbinol derived from *N*-methylnicotinamide. A substance claimed to be identical with the natural product has been prepared from nicotinamide methiodide. *iso*Butanol was used to extract F_2 from urine, and Najjar *et al.* (1944) have suggested that the solvent and the carbinol may form an ether which, they claim, explains the slow increase in fluorescence of *iso*butanol extracts. We have used alcohols (ethylene glycol and *n*-butanol) for extraction and are aware of the danger of artifact production by similar means.

The substance F_2 must not be confused with the substances present in normal urine which fluoresce without any treatment. F_2 is derived from a non-fluorescent precursor which is rendered fluorescent by making the urine alkaline and extracting with *iso*butanol. It is well known that nicotinamide readily undergoes biological methylation in certain species (including man) and that pyridinium compounds are formed. Such compounds can be reversibly oxidized and reduced.



The reduced or dihydro form in such a system exhibits strong fluorescence when irradiated with

ultraviolet light whilst the oxidized form does not (Morton, 1942). Our extracts behave in exactly the opposite way; the fluorescence is destroyed by reduction with dithionite and restored on reoxidation with air or H_2O_2.

We observed that normal human urine fluoresces blue, and that this fluorescence (not due to F_2) is reversibly destroyed by dithionite, whereas bromine water or acid permanganate causes its irreversible destruction. In addition, according to Ellinger & Coulson (1943), samples of F_2 obtained by them absorbed selectively in the ultraviolet at 271, 306 and 347 mμ. We have therefore no evidence to suggest our extracts contain F_2. There are, however, certain similarities between some fluorescing substance in urine and our extracts which justify further investigation.

Dickson, Forziati & Schoonover (1952) and Dickson, Forziati, Lawson & Schoonover (1952) have utilized the natural fluorescence of teeth in investigating the structure of enamel and dentine. Their photomicrographs show that the intensity of fluorescence appears to be an indication of the relative organic content of the various areas of the tooth. In dentine they report that fluorescence is concentrated along the dentinal tubules and the dentino-enamel junction. The fluorescent materials in our extracts are undoubtedly breakdown products of the organic material existing in the tooth. We are less certain that the mineral phase is devoid of fluorescence for, after repeated extractions with alkaline ethylene glycol, the mineral residue still fluoresced slightly; this may of course be due to traces of organic material not extracted by our treatment.

Reeder & Nelson (1940) have studied the fluorescence associated with proteins. When examined in ultraviolet light of wavelength 310–410 mμ. they found that many proteins gave a bluish white fluorescence in the solid state and a more green fluorescence in solution. Acid hydrolysis increased the fluorescence of those proteins containing tryptophan much more than in the case of those deficient in this amino-acid. Tyrosine and tryptophan were the only amino-acids which gave blue-green fluorescence with glucose when boiled with dilute hydrochloric acid. We are now investigating the fluorescence of various proteins and comparing it with that of the teeth and tooth extracts.

The preliminary investigations reported here have merely cleared the ground a little and have enabled us to define the problem in more adequate terms. We can conclude that the substances responsible for the fluorescence in our extracts are organic compounds apparently present in very small amounts. They contain, or are closely associated with, groups capable of acetylation or methylation. It is of interest to have found some evidence of substances which can undergo reversible oxidation and reduction, but until we have obtained more information we cannot speculate on the nature of any oxidation-reduction system. There must also be a group present which absorbs in the region of the wavelength of the activating radiation (about 366 mμ.). So far we have been unable to detect any selective absorption at this longer wavelength owing to the presence of continuous absorption by the impurities in our extracts. Irradiation with monochromatic ultraviolet light of shorter wavelength may of course reveal additional fluorophoric groups. Our next task is to obtain the fluorescent extracts in a state of greater purity. Although we have isolated many fluorescing fractions, we cannot ignore the possibility that we may be dealing with the same fluorophoric grouping attached to a variety of carriers, which would account for the differing chromatographic behaviour of our samples. The apparent complexity of the problem may therefore be due to our own experimental methods.

SUMMARY

1. Digestion with alkaline ethylene glycol or alkaline *n*-butanol extracts much of the fluorescent material from dentine, that is it is removed along with the organic fraction.

2. By the use of adsorption chromatography on alumina columns many fluorescent fractions have been obtained.

3. Most of the fractions do not exhibit any selective absorption in the ultraviolet region.

4. Some fluorescent material is present in the extract after sulphuric acid digestion.

5. The fluorescence of the dentine extract is due in part to organic substances other than amino-acids.

6. We have some evidence that our extracts contain a fluorescent pigment which can undergo reversible oxidation-reduction, but no evidence that xanthopterin, riboflavin or nicotinamide derivatives are present.

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Chondroitin Sulphuric Acid as a Factor in the Stability of Tendon

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The intercellular substances of connective tissue consist of fibrillar and amorphous components. On morphological grounds the histologists have postulated the presence of an amorphous substance cementing collagen fibrils together into fibre bundles, and an amorphous ground substance in which these bundles are embedded (Maximow & Bloom, 1948). Meyer and his co-workers (Meyer & Smyth, 1937; Meyer & Chaffee, 1941) have isolated the mucopolysaccharides chondroitin sulphuric acid (CSA) and hyaluronic acid from skin, cartilage and tendon, and these substances have been identified with the cement and ground substances, respectively. However, apart from the work of Meyer, Palmer & Smyth (1937) on protein-CSA complexes, of Partridge (1948) on cartilage and of Einbinder & Schubert (1951) on the binding of CSA by collagen, little information is available concerning the association of the mucopolysaccharides and collagen.

Rat-tail tendon can be dissolved in dilute acetic acid (Nageotte, 1927), and from the solution fibres can be reconstituted by dialysis and salt precipitation (Huzella, 1932; v. Buzagh, 1942), and by addition of mucopolysaccharides (Highberger, Gross & Schmitt, 1951). When examined under the electron microscope fibrils precipitated with 0·7–1·5 % (w/v) potassium chloride showed the characteristic 640 A. spacing (Bahr, 1950). At these concentrations, needle-shaped crystals or tactoids were produced, having the striated structure of collagen, while outside this range (0·5–10 %) long, non-striated, fibrils were formed (Vanamee & Porter, 1951). Mucopolysaccharide-precipitated fibrils were found to have a wide spacing of up to 2000 A., although small concentrations of serum mucoprotein gave the 640 A. spacing (Highberger *et al.* 1951). This was also shown for heparin-precipitated fibrils (Morrione, 1952). However, CSA-precipitated fibrils were either non-striated or had spacings up to 2000 A. (Highberger *et al.* 1951).

In these studies little emphasis was placed on obtaining mucopolysaccharide-free collagen, the tissue being usually extracted with 10 % (w/v) sodium chloride or 30 % potassium chloride. Partridge (1948) and Woodin (1952) showed that extraction with 10 % (w/v) calcium chloride was necessary to remove all the mucopolysaccharide, and this method involved the solution of protein that appears to be of collagenous origin.

Before studying the relationship of mucopolysaccharide to collagen, it seemed essential to obtain mucopolysaccharide-free collagen by other methods. It has been reported (Meyer, Chaffee, Hobby & Dawson, 1941; Madinaveitia & Stacey, 1944) that