The Histochemical Demonstration of Keratin by Methods involving Selective Oxidation

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With two plates (figs. 1 and 2)

SUMMARY

1. Oxidation of tissues with performic acid gives rise to histochemically detectable reaction products particularly in two classes of material. These are keratin and lipoids of the phosphatide class.

2. Three methods have been evolved for visualizing the effect of performic acid on cystine-containing structures; two of these (performic acid/Schiff and performic acid/cobalt nitrate) also record the effect on lipoids.

3. An attempt has been made to elucidate the chemistry of the reactions and it is suggested that oxidation of cystine in the tissues gives rise not only to cysteic acid (alanine-beta-sulphonic) but to another acid (alanine-beta-sulphinic). The latter is responsible for the positive reaction with Schiff's solution.

4. The Schiff reaction with performic acid oxidized lipoids is due to the formation of substances giving the reactions of aldehydes. It is possible that similar groups may be produced from lipoid molecules by periodic acid oxidation and that these and not polysaccharides (1·2 glycols) are responsible for the periodic acid-Schiff reaction in such cases.

A HISTOCHEMICAL reaction for keratin has long been needed and investigations have been carried out, during the past 18 months, with the object of finding a satisfactory test. These have involved the oxidation of tissue sections with a number of different reagents. The disulphide bond of cystine is well known to be especially reactive and a variety of oxidizing agents act preferentially upon it. According to Smith and Harris (1936), hydrogen peroxide slowly oxidizes cystine to cysteic acid (alanine-beta-sulphonic acid, $HO_3S.CH_2.CH(NH_2).CO_2H$) and this substance has also been identified in the reaction products from treatment of wool by halogens such as fluorine (Hudson and Alexander, 1946), and chlorine or bromine (Consden, Gordon, and Martin, 1946). Alexander, Hudson, and Fox (1950), using potassium permanganate in 0·5 N sulphuric acid, succeeded in oxidizing 30 per cent. of the cystine present in horn keratin to cysteic acid and other products, and these authors found that if peracetic acid was used for oxidation as much as 75 per cent. of the cystine was oxidized in a period of 2 hours. In this case cysteic acid was considered to be the only product of the reaction.

Whatever the oxidizing agent employed, the main histochemical problem has been to demonstrate and identify the reaction products. In the early stages

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of the investigation oxidation by perchloric acid and periodic acid were followed by treatment with cobalt salts in the hope that if sulphides were released as a preliminary stage to oxidation these might be converted to the black CoS. Since this hope was not fulfilled the cobalt-treated slides were subsequently immersed in dilute yellow ammonium sulphide and cobalt combined in colourless form in the tissues was visualized in this manner. Using this method, after either perchloric or periodic acid, weakly positive results were obtained in the hair shafts and often much stronger results in the layers of Huxley and Henle in the hair root sheath. The publication by Dempsey, Singer, and Wislocki (1950) of a paper on the increased basiphilia of the tissue proteins after oxidation with periodic acid drew attention to the possibility of demonstrating the oxidation products of cystine by means of dilute methylene blue solutions at low pH levels. Using this dye at pH 4·9, these authors observed that after periodic acid oxidation the most intense basiphilia was found in the skin and hair, and in the pancreatic islets and endothelia of small blood-vessels, all regions known or presumed to have a high content of sulphur-containing amino-acids. They suggested that oxidation of SH and S.S groups leads to the formation of sulphonic acids and that these, being relatively strongly dissociated, were responsible for the observed increase in basiphilia. In my own investigations the results obtained with H_2O_2, $KMnO_4$, and with perchloric and periodic acids, were unsatisfactory as a test for keratin in tissue sections, even when they were used in conjunction with the methylene blue method. Peracetic and performic acids were therefore tried.

Toesnies and Homiller (1942) showed that performic acid reacts only with tryptophan, cystine, and methionine among the amino-acids, whereas, according to Nicolet and Shinn (1929), periodic acid attacks tryptophan, methionine, cystine, and the alpha-hydroxy amino-acid serine. In the case of performic acid the oxygen consumption figures for cystine correspond to its conversion to cysteic acid while, according to Alexander, Hudson, and Fox (1950), partition chromatography reveals that only with peracetic oxidation does the reaction product consist entirely of cysteic acid. Since theoretical considerations indicated that a major portion, at least, of the reaction product was likely to be a sulphonic acid, and since neither methylene blue nor cobalt salts could be considered in any way specific for sulphonic acids, some other way of demonstrating these was sought. A method for their identification *in vitro* was suggested by Latimer and Best (1937), which involved the formation of phenylhydrazides, but the colour of these is too faint to be visible in tissue sections. An aryl hydrazide, 2-hydroxy-3-naphthoic acid hydrazide (NAH), was therefore used instead. With this compound visualization was achieved by coupling the sulphonhydrazide, formed in the tissues, with diazotized diorthoanisidine. The colour obtained by this process, derived from the ketosteroid methods of Camber (1949) and Ashbel and Seligman (1949), was a deep purplish blue. A reddish colour is given by structures with which the hydrazide combines by virtue of other groups in its molecule and

such a colour does not constitute a positive result. Using this NAHD technique the best results were obtained when oxidation of sections was carried out with performic acid (fig. 1A) and, since NAHD had been usefully employed as an aldehyde reagent (Pearse, 1951), Schiff's solution was automatically suggested as an alternative. In the case of hair-shaft structures the results with Schiff's solution were as good as those obtained by the use of dilute methylene blue at pH 2·6. In each case these structures were unstained in control sections. If the pH of the methylene blue solution was raised, a general increase in tissue basiphilia was observed, as found by Dempsey and his associates.

Material and Methods

The material examined consisted of human and rat skin and sections of a human teratoma (dermoid cyst) of the ovary, fixed in alcohol, formalin, formaldehyde/mercuric chloride, Helly, Bouin or Carnoy, or in boiling chloroform-methanol. The epidermal structures of a teratoma were examined because the cystine content of foetal skin is known to be higher than that of the adult. The three methods used, and a variation of one of them, are given below.

Performic acid/Schiff (PFAS)
Performic acid/methylene blue
Performic acid/cobalt nitrate

For all three methods:

1. Bring paraffin sections to water, with removal of mercury precipitate where necessary.
2. Treat with performic acid solution (A) for 10 minutes.
3. Wash in running water for 2–5 minutes.

After this stage there are three alternatives.

PFAS Method I

4. Immerse in Schiff's solution (B) for 30 minutes.
5. Wash in warm running water for 10 minutes.
6. Dehydrate in the alcohols, clear in xylene, and mount in D.P.X.

Counterstaining of the nuclei may not be made by means of basic dyes since the basiphilia of the tissues is enormously increased.

Alternatively (*PFA/methylene blue*),

4. Immerse in M/2000 methylene blue in 0·1 M veronal acetate buffer at pH 2·6 for 2 hours.
5. Wash briefly in distilled water.
6. Dehydrate rapidly in the alcohols, clear, and mount as above.

Alternatively (*PFA/cobalt nitrate*),

4. Treat with 2 per cent. aqueous cobalt nitrate for 5 minutes, wash, and

5. Treat with dilute yellow ammonium sulphide (1 drop in 20 ml. of water) for 5 minutes.
6. Wash and counterstain nuclei with carmalum if desired (3–6 hours).
7. Dehydrate, clear, and mount as above.

PFAS Method II

1. Bring paraffin sections to water.
2. Cover the slides with a mixture of equal parts of performic acid solution (A) and Schiff's solution (B), for 2–5 minutes.

The mixture is unstable and must be freshly prepared. A dirty brownish red colour develops, which may be ignored.

3. Wash in hot running water for 10 minutes.
4. Dehydrate, clear, and mount as above.

Solution A (Performic acid)

Add to 40 ml. of 98 per cent. formic acid 4 ml. of 30 per cent. (100 vol.) H_2O_2. Allow the mixture to stand for at least 1 hour before using and use preferably within 24 hours. More performic acid may be developed by adding a further 4 ml. of H_2O_2, but dilution of the formic acid solution beyond this point is not advisable since Feulgen hydrolysis effects begin to appear in the nuclei when weak acid hydrolysis is followed by Schiff's reagent.

Peracetic acid was made by adding 20 ml. of acetic anhydride to 5 ml. of 30 per cent. H_2O_2. After thorough mixing, and standing for 2 hours, the solution was diluted to 50 ml. before use.

Solution B (Schiff's reagent)

This was prepared according to the directions of de Tomasi (1936) and used at full strength.

Naphthoic acid hydrazide-dianisidine (*NAHD*)

This method was used essentially as described by Ashbel and Seligman (1949). Sections were treated at 22° C. for 3–5 hours with a 0·1 per cent. solution of 2-hydroxy-3-naphthoic acid hydrazide in 50 per cent. ethanol with 5 per cent. acetic acid. They were then washed in 50 per cent. ethanol (two changes) for 1 hour and treated with a freshly prepared diazotate of o-dianisidine, or with the stable diazotate (Brentamine fast blue Salt B), in 0·1 M veronal acetate buffer at pH 7·5 and at 4° C. After washing in water, dehydration, clearing, and mounting were performed as above.

Results

Fig. 1B illustrates the effect of the cobalt nitrate and ammonium sulphide method on hair-follicle structures after oxidation with performic acid. The hair shafts themselves, which usually appear pale grey, have fallen out in this example, but in both follicles the staining of the Huxley-Henle layers is demonstrated. Fig. 1 C–E illustrates the application of methylene blue at pH 2·6 after performic acid oxidation. In fig. 1C only the hair shafts and the

stratum corneum stain deep blue, while in fig. 1D the cortex of the hair shaft is revealed as staining more strongly than the medulla. The Huxley-Henle layer, which photographs because it is refractile, remains quite unstained. Fig. 1E shows the same points in a longitudinal section. Figs. 1F and 2A illustrate the performic acid/Schiff method I, and fig. 2B method II. Once again the hair shafts and stratum corneum stain strongly (fig. 1F) but, in addition, the Huxley-Henle layers are also stained (fig. 2 A and B). In fig. 2B only the darkly stained structures are red in colour, the others appear in the photograph solely on account of their refractile qualities.

In view of the positive reaction obtained with both Schiff's solution and NAHD, after performic acid oxidation, the possibility of aldehyde formation was considered. It was thought that the reaction of cobalt salts with the various oxidized structures might be due to their combination with strongly dissociating sulphonic acids or, alternatively, to oxidation of aldehydes by the cobalt salt. Such oxidation occurs with a variety of heavy metals (Karrer, 1950). In applying the PFAS method I to various tissues it had been noted that, in addition to structures which could be presumed to contain cystine, certain others also reacted strongly. Notable among these were the red cell envelopes and myelin sheaths together with various intracellular granules of known lipoid constitution. Verne (1929) showed that mild oxidation of fatty substances, such as lecithin, for instance, would produce groups having all the reactions of aldehydes and it seemed probable that a similar mechanism was responsible for the positive PFAS reaction in known lipoid-containing structures. Various experiments were carried out in order to test both this hypothesis and also the suggested mechanism for performic acid oxidation of tissue cystine. Table I, below, gives the reactions obtained in the case of the tissue components listed, in paraffin sections. Since formic acid is certainly,

TABLE I

	Tests				*Controls*	
	Performic acid				*Formic acid*	H_2O_2
Structure	*Schiff*	*NAHD*	*Meth. blue pH 2·6*	*Ammoniacal silver*	*Schiff*	*Schiff*
Red cell envelope	+	+	−	+	+	−
Hair shaft	+	+	+	−	−	−
Myelin sheath	+	+	−	+	weak +	−

and free H_2O_2 probably, present in the performic acid solution used in these reactions, control sections were treated with the two reagents separately. With 10 per cent. H_2O_2 negative results were obtained but with 98 per cent. formic acid a weakly positive reaction occurred in myelin sheaths and a stronger one in the red cell envelope. The reaction with myelin was entirely

reversed by fixation in boiling chloroform-methanol for 8–16 hours, but traces of a positive reaction remained in the red cells even after this procedure, which is presumed to remove all but the most closely bound lipoid from small blocks of tissue. It can be seen from fig. 2C that the Huxley-Henle layers of the root sheath react positively with Schiff's solution after formic acid alone, though to a lesser degree than with PFAS method I. If the simultaneous method II is employed, using equal parts of formic, or performic, acid and Schiff's solution, no reaction occurs in any tissue component with formic acid/Schiff, but with PFAS the hair shafts give a brilliant reaction and the Huxley-Henle layers a weaker one (fig. 2B). All other structures except the stratum corneum are unstained. Table I shows that both red cell envelopes (fig. 2D) and myelin react with ammoniacal silver solutions after performic acid oxidation, while hair shaft and keratin structures do not. According to Cain (1949) aldehydes are the reducing groups most likely to be present in oxidized lipoids, and the reduction of ammoniacal silver salts, though it is by no means specific for aldehydes, was considered to have this significance. The peroxides and hydroperoxides, which Cain considered to be responsible for the plasmal reaction of certain lipoid structures, should give a positive reaction with the nadi reagent (α-naphthol and dimethyl-*p*-phenylenediamine). This effect was not produced by performic acid/oxidized sections. The positive Schiff reaction after formic acid might be due to the presence of this acid in bound form or to the presence of formates. Although it is usually suggested (Karrer, 1950) that formic acid and its salts can act as aldehydes by simple molecular rearrangement, Haas (1951) observed that formates gave none of the usual aldehyde reactions and he considered that their action in reducing ammoniacal silver solutions was due to the ease with which formic acid parts with its two hydrogen atoms.

Table II shows the results of applying performic acid, and its constituents separately, to Schiff's reagent in the proportions indicated. It also shows the effects of cystine, methionine, arginine, and tryptophan on the oxidation of Schiff's reagent *in vitro*. These results suggest that if traces of formic acid or H_2O_2 remain in the sections they will not give rise to a positive Schiff reaction.

TABLE II

Schiff's reagent and	*Effect*
Performic acid (10:1)	No recolorization (10 min.).
Performic acid (1:1)	Increasing red colour (oxidation).
Formic acid (5:1 and 1:1)	No recolorization.
H_2O_2 (5:1)	No recolorization.
Performic acid/cystine (1:1)	No recolorization (S.S prevents oxidation of Schiff).
Performic acid/methionine (1:1)	No recolorization (SH prevents oxidation of Schiff).
Performic acid/arginine (1:1)	Increasing red colour (no prevention of oxidation).
Performic acid/tryptophan (1:1)	Faint pink (1 hour).

Performic acid might conceivably do so but, since only a trace will be present, with excess of Schiff's reagent, this is not considered likely. As mentioned above, both performic and periodic acids are known to oxidize tryptophan, one of the products being indole-3-aldehyde, and the faintly positive result, which is difficult to see through the brown of oxidized tryptophan, suggests that this amino-acid may be concerned in the genesis of colour with Schiff's reagent when oxidizing agents are applied to the tissues. The immediate oxidation of Schiff's solution *in vitro* is prevented by excess of either cystine or methionine and the products in the latter case give no recolorization on standing. With cystine, however, after an hour or more, the solution becomes bluish purple. Arginine, and other amino-acids which are not attacked by performic acid, entirely fail to prevent recolorization. Taking the above observations into account, figs. 1 C–F and 2A and B show (1) that the product of the reaction between keratin and performic acid remains at least partially at the site of production, (2) that it contains an acid, sufficiently strong to dissociate at pH 2·6, and (3) that it contains a substance which recolorizes Schiff's solution. The first two criteria would be satisfied by a sulphonic acid, and such a substance would also combine with NAH, but sulphonic acids are not known to react with Schiff's solution. When this point was investigated by mixing *p*-toluenesulphonic acid with Schiff's reagent *in vitro*, no colour was produced. The corresponding *p*-toluenesulphinic acid, however, produced a strong but slowly developing reaction under the same circumstances. This finding suggests that the oxidation of cystine in tissue sections produces not only alanine-beta-sulphonic acid but also, perhaps as an intermediate stage, alanine-beta-sulphinic acid ($HO_2S.CH_2.CH(NH_2).CO_2H$). *In vitro*, oxidation of cystine to cysteic acid is observed to take place without giving a Schiff-positive reaction product in the early stages.

The experimental evidence so far offered suggests that in performic acid/oxidized sections three possible groups may account for the appearances observed when these are subsequently treated with Schiff's solution, methylene blue, or cobalt salts. The three groups are sulphonic (SO_3H), sulphinic (SO_2H), and aldehyde. In order to establish responsibility more accurately the reactions concerned were carried out *in vitro* and by a modified Coujard technique (Coujard, 1943). In the latter the various reagents are dissolved or suspended in a serum-gelatin mixture and used to draw lines or bands on the surface of a glass slide. The slides are subsequently dried and exposed to formalin or formalin vapour for 6–18 hours. They are well washed in running water before use in histochemical reactions. The results of these manœuvres appear in Table III. It is clear that both sulphonic and sulphinic acids may be responsible for the reaction with methylene blue and possibly with Schiff's reagent also, since in this case some colour was produced by sulphonic acid in the Coujard technique. Aldehydes are not responsible for the results with methylene blue but might be responsible for some of the colour both with Schiff and the NAHD reagent. It was observed that the aldehyde-blocking reagents dimedone (saturated solution in 5 per cent. acetic alcohol)

TABLE III

	In vitro		*Coujard*			
Radicle	*Schiff*	*Ammoniacal silver*	*Schiff*	*NAHD*	*Ammoniacal silver*	*Meth. blue pH 2·6*
SO_3H *p*-toluene sulphonic	−	−	faint +	+	−	+
SO_2H *p*-toluene sulphinic	+	−	+	faint +	−	+
CHO phthaldehyde	+	+	+	+	+	−

and phenylhydrazine (2 per cent. solution in 5 per cent. acetic alcohol) reversed the reactions of performic acid/oxidized red cells. Phenylhydrazine (60° C., 24 hours) greatly reduced the Schiff reaction of oxidized hair shafts, but dimedone, acting for a short period, had not this effect. A similar reduction was produced by acetic alcohol alone, however, which indicates solubility of the reaction product rather than specific blocking. These experiments suggest that aldehydes are produced by performic acid oxidation of lipoids but they do not establish this fact. Provided that it is used at a low enough pH the methylene blue technique is seen to be more specific than the other two methods for the demonstration of sulphur-containing reaction products.

DISCUSSION

From the experimental work which has been carried out it is suggested that performic acid oxidation, followed by acid methylene blue or by Schiff's reagent, can demonstrate the presence of cystine in keratin. In the case of the hair shafts, which may contain up to 17 per cent. of cystine, the reaction is brilliant and unequivocal. In the stratum corneum of the skin and in the epithelial pearls of keratinizing tumours the results are much less striking. Furthermore, since in these latter sites, and in the Huxley and Henle layers, a positive Schiff reaction can be produced by substituting formic for performic acid, there is reason to suppose that part of the latter reaction is due to production of aldehyde groups from those lipoids which can be shown to be present by other techniques. While the sulphinic acid/Schiff compound is insoluble in alcohol, and withstands the process of dehydration, the aldehyde-Schiff compound, after both formic and performic acids, is partly soluble in alcohol. Dehydration therefore increases the contrast between the two forms. The usefulness of the PFAS technique for the diagnosis of acidophil proteins as keratin is limited by the often weak character of the reaction in other than hair components, and the same objection applies to the methylene blue method. It is sometimes easier to assess the results of oxidation if the cobalt nitrate method is used (fig. 2E), while with other examples of material sup-

posedly keratin, PFAS gives very adequate results. Fig. 2F shows the intra- and extracellular masses of protein in a plantar wart, stained by the PFAS method; the result suggests that both contain keratin.

Four possible factors may explain the lower intensity of the reaction with all structures other than hair shafts.

1. Such structures contain less cystine. This is certainly true but the difference could hardly be expected to account for the very great difference observed with the PFAS reaction.
2. The fainter-staining structures may contain their sulphur largely in the form of sulphydryl (cysteine). This substance is more easily oxidized than cystine and thus the suggested intermediate product (alanine-beta-sulphinic acid) might be absent. Cysteic acid (alanine-beta-sulphonic acid) should be demonstrable by either of the alternatives to Schiff's solution employed, yet these do not always give satisfactory results.
3. In the case of the fainter-staining structures the reaction products may be more diffusible. The products of performic acid oxidation of cystine *in vitro* diffuse freely into the supernatant. That this diffusion is limited in the case of tissue sections is perhaps due to linkage of the reaction products in the polypeptide chain of keratin or to their combination with other components of the keratin molecule.
4. Stereochemical factors diminishing the availability of SH or S.S for oxidation may be concerned.

Which, if any, of the above factors is responsible for the results observed in practice has not yet been determined, and the specific location of the sites of cystine oxidation remains to be proved. Except for its additional effect on certain lipoids, performic acid does not give rise to any other groups reacting with Schiff's solution although it causes a general increase in the basiphilia of oxidized tissues such as Dempsey, Singer, and Wislocki found with periodic acid oxidation. In contrast to periodic acid, however, it has no effect on the 1·2 glycol group, so that mucopolysaccharides, mucoproteins, and glycogen remain unstained by the techniques here described. Two points of interest remain to be considered. First, the demonstration of keratin would undoubtedly be improved if a more suitable chromogenic agent could be found to combine readily with sulphonic and sulphinic acids and not with other basiphilic groups such as COOH, for instance, which are produced in the tissues by oxidations of the type we have been considering. Secondly, certain of the lipoid components observed to be PFAS positive are also positive by the PAS technique of McManus (1946) and Hotchkiss (1948). Interpretation of the latter reaction, in the case of such components, may have to be reorientated in favour of a lipoid rather than a polysaccharide (1·2 glycol) origin for the aldehyde groups responsible for the recolorization of Schiff's solution.

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DESCRIPTION OF PLATES

FIG. 1. A. Transverse section of hair follicle. Hair shaft stained purplish blue, the other structures reddish. Performic acid, naphthoic acid hydrazide, diazotised dianisidine. ×250.

B. Two hair follicles in transverse section. In both, the layers of Huxley (especially) and Henle are stained dark grey. Performic acid, cobalt nitrate, carmalum. ×100.

C. Epidermal structures in a dermoid cyst. Only keratin in hair and stratum corneum stain dark blue. Performic acid, M/2000 methylene blue at pH 2·6. ×75.

D. Transverse section of hair follicle. The cortical layer of the hair stains dark blue, the medulla to a lesser extent. No other structure stains positively. Performic acid, M/2000 methylene blue at pH 2·6. ×240.

E. Longitudinal section of hair follicle. The outer portion of the hair shaft (cortex and medulla) stains dark blue. Melanin granules stain in control section also. Performic acid, M/2000 methylene blue at pH 2·6. ×100.

F. For comparison with C. Keratin in hair and stratum corneum stained bright magenta-red. PFAS method I. ×75.

FIG. 2. A. For comparison with fig. 1D. The hair shaft and Huxley's layer both stain bright magenta-red. PFAS method I. ×360.

B. For comparison with fig. 1E. The outer portion of the hair shaft and Huxley's layer stain bright magenta-red. PFAS method II. ×50.

C. Transverse section of hair follicle. Hair shaft unstained. The Huxley and Henle layers stained magenta-red, 98 per cent. formic acid, Schiff's reagent. ×200.

D. Blood-vessel with large numbers of red cells stained black. Performic acid, ammoniacal silver. ×80.

E. Keratin masses for a squamous cell carcinoma, secondary in a lymph node. Performic acid, cobalt nitrate, carmalum. ×100.

F. Plantar wart. Below, large keratin masses, and above, intra- and extracellular inclusions. PFAS method I. ×47.

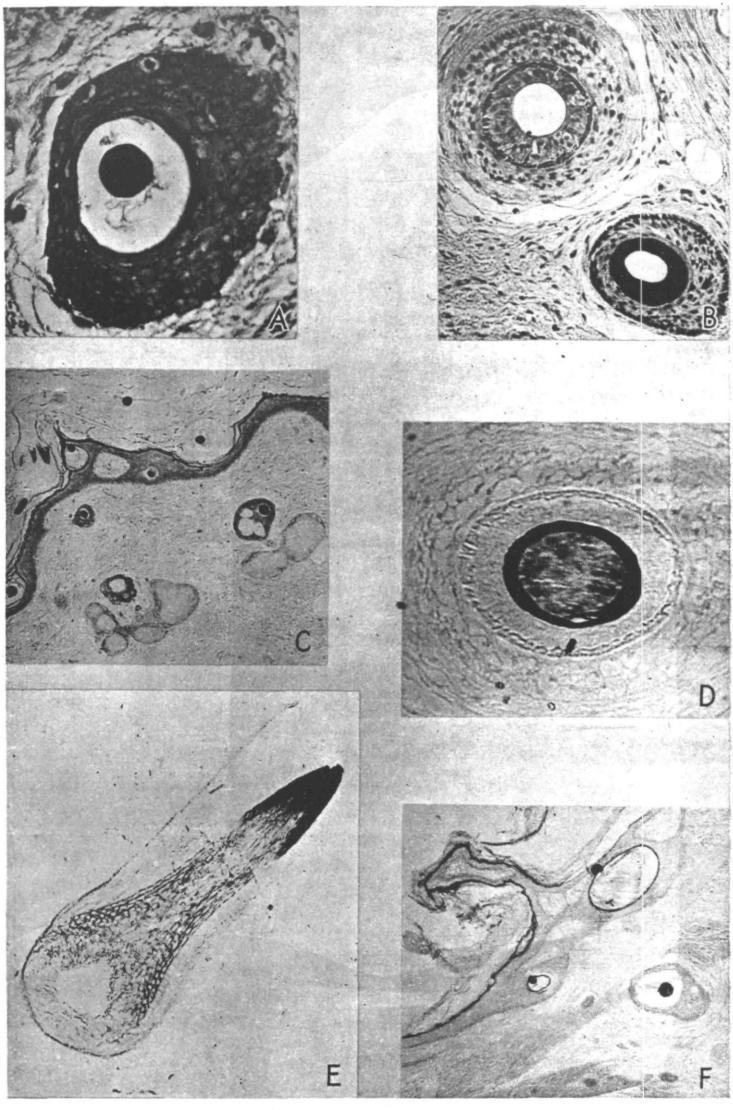

FIG. 1

A. G. E. PEARSE

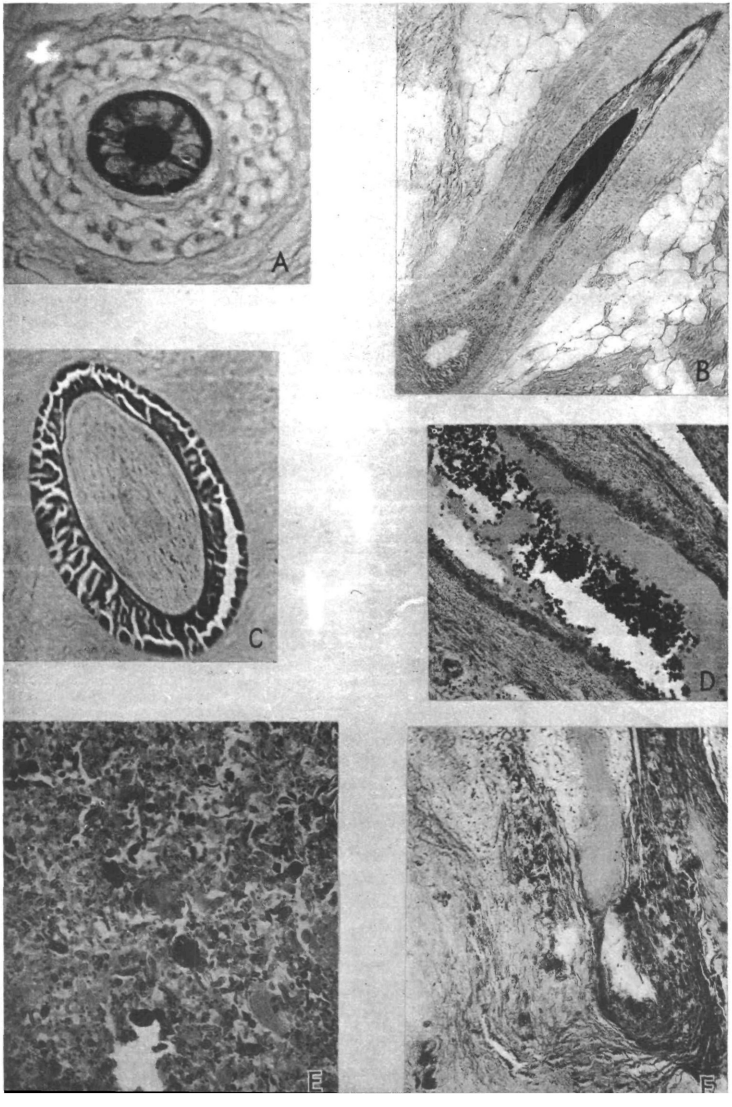

FIG. 2.

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