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STUDIES ON THE ISOLATION AND COMPOSITION OF INTERSTITIAL FLUID IN SWINE DENTINE

by

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All tissues contain interstitial fluid which is, at least partly, an ultrafiltrate of blood. The oldest description of an interstitial fluid in dentine known to us is that given by *Kölliker* in his textbook of anatomy (1852). Active interest in the interstitial fluid of dentine began when *Spreter v. Kreudenstein* (1955) developed the first practicable methods for its isolation and started his pioneer studies on its composition.

The observations of *Heikinheimo* (1963) on the movements of dentine interstitial fluid (DIF) under pressure prompted us to try to isolate DIF by centrifugal force. The purpose of this paper is to describe the method and some results of the analysis of the fluid so isolated.

MATERIAL AND METHODS

Pigs' teeth, which have been used previously in studies of DIF (*Spreter v. Kreudenstein & Stüben,* 1956), were chosen as test objects. The teeth were obtained from a slaughterhouse, where they were extracted from adult pigs just after death. The bifurcation area of the tooth was removed beneath the crown, the root cut off, and the pulp excavated. The odontoblast layer and the remnants of the pulp were removed with a water-cooled drill at low revolutions and low pressure. The enamel and cement were not removed.

The prepared teeth were placed in metal centrifuge tubes with the dentine canals directed towards the bottom of the tube. A refrigerated centrifuge (MSE MR 65, Measuring & Scientific Equipment Ltd., London), was used (16,000 r.p.m., 50 min., temperature + 4° C). The fluid which flowed out was collected from the bottom of the tube. If the DIF was not isolated immediately after preparation, the teeth were stored frozen. Blood samples for preparation of serum were taken from the jugular vein of the pigs just after death.

For the investigations samples of DIF from different adult pigs were pooled. The following analyses were made on both DIF and serum.

The nitrogen content was determined by Kjeldahl combustion and subsequent distillation of the ammonia with a Parnas-Wagner apparatus. Non-protein nitrogen was determined according to *Folin* (1930). The haemoglobin was estimated by the cyanmethaemoglobin method according to *Chilcote & O'Dea* (1953). The hexosamines were determined according to *Blix* (1948), and uronic acids by a modification of the carbazole method (*Bitter & Muir*, 1962) and by the orcinol reaction according to *Dische* (1955). The sodium content was determined with a flame photometer (No. 866150, Evans Electroselenium Ltd., London). Hydroxyproline determinations were made according to *Stegemann* (1958) and *Woessner* (1961).

Mucopolysaccharides were isolated after hydrolysis with trypsin (Trypure®, Novo, Copenhagen) for 24 h. at + 37° C in ammonium bicarbonate buffer, pH 8.3, or with papain (E. Merck AG, Darmstadt) for 24 h. at + 65° C (*Scott*, 1960; *Buddecke*, 1960). The mucopolysaccharides were precipitated from the hydrolysate with a 4-fold volume of ethanol and the precipitate dissolved in a small volume of distilled water. Acid mucopolysaccharides (AMPS) were isolated by precipitating with an excess of 1 per cent aqueous solution of cetylpyridinium chloride (Recip AB, Stockholm). The precipitate was centrifuged and dissolved in 2*N* $MgCl_2$ (*Scott*, 1960). The AMPS were reprecipitated several times with 4-fold volumes of ethanol and dissolved in distilled water.

Electrophoresis of AMPS was performed, using a cellulose acetate membrane (Oxoid®, Courtaulds Ltd., Coventry) in barbi-

turate buffer, pH 8.65, $\mu = 0.125$, 110 V potential, and stained with a 1 per cent solution of Alcian blue in 25 per cent acetic acid (*Näntö,* 1963). Prior to electrophoresis some samples of AMPS were incubated with desoxyribonuclease (Calbiochem, Los Angeles) in order to remove any contaminating desoxyribonucleic acid (3 hours, tris-buffer pH 7.2, + 37° C).

Electrophoresis of proteins was carried out for 3 hours in the same conditions and the strips were stained with 0.005 per cent nigrosin in 20 per cent acetic acid or with 0.3 per cent lissamine green in 15 per cent acetic acid. Glycoproteins were demonstrated with periodic acid-Schiff staining by the method of *Aronson* (1961) as modified by *Frey* (1964).

Electrophoresis of proteins in starch gel was performed with a discontinuous buffer system (*Poulik,* 1957), 200 V, and 6 hours, and the gels were stained with 0.25 per cent Amido Black 10 T (90 per cent methanol, 10 per cent acetic acid).

The cellulose acetate strips were examined with a Beckman Model B spectrophotometer with an attachment for densitometry constructed in our laboratory.

RESULTS

Isolation of DIF

About 0.01 ml of DIF per gm of prepared tooth was obtained. Usually the colour of the fluid was pink and its haemoglobin content was approximately 0.17 g per 100 ml. Since the haemoglobin content of swine blood was approximately 13.8 g per 100 ml, the DIF contained about 1.2 ml of blood per 100 ml.

Results of the chemical analyses are given in Table 1.

The electrophoretic analyses

Figure 1 A shows the electrophoretic pattern of DIF proteins on cellulose acetate. The relative concentration of albumin was 36 per cent in DIF and that of the globulins 64 per cent. In the serum these values were 54 and 46 per cent, respectively. Both nigrosin and lissamine green staining gave the same relative concentrations for albumin and globulins. However, both qualitative and quantitative differences were found in the glycoprotein patterns revealed by periodic acid Schiff staining (Fig. 1 B).

Table 1

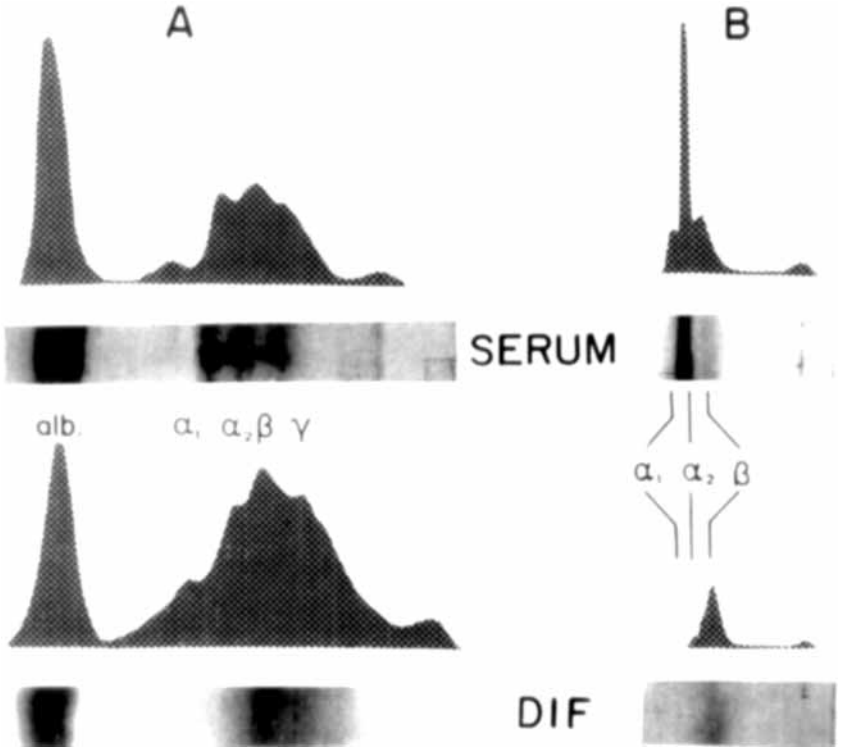
The results of the chemical analyses. The values for the acid mucopolysaccharides are given as mg per 100 ml original DIF.

	DIF	Acid mucopolysaccharides of DIF	Swine serum
Nitrogen	246.4 mg/100 ml	—	986.0 mg/100 ml
Protein, calc. (6.25×N)	1.54 g/100 ml	—	6.14 g/100 ml
Non-protein nitrogen	28.7 mg/100 ml	—	—
Haemoglobin	0.17 g/100 ml	—	(whole blood) 13.8 g/100 ml
Hydroxyproline	13.69 mg/100 ml	—	3.24 mg/100 ml
Hexosamines	46.7 mg/100 ml	2.70 mg/100 ml	77.8 mg/100 ml
Uronic acids			
– carbazole	—	2.60 mg/100 ml	—
– orcinol	—	5.80 mg/100 ml	—
Ratio of carbazole to orcinol	—	0.45	—

In starch gel electrophoresis also the protein pattern of DIF seemed to differ from that of swine serum. The presumable haptoglobin fractions*) of DIF were relatively stronger and some of the fractions which were visible in the serum protein pattern could not be detected in DIF. They were not identified further (Fig. 2).

Using Alcian blue staining we found two positively staining fractions in cellulose acetate electrophoresis, which moved to the anode ahead of the protein fractions (*Kulonen et al.*, 1964). When AMPS of DIF were isolated using hydrolysis with proteolytic enzymes and precipitation with cetylpyridinium chloride, three Alcian blue-positive fractions were found on cellulose acetate. The mobilities of the two fastest fractions corresponded to those of chondroitin sulphate and hyaluronic acid, respectively (Fig. 3). To remove the desoxyribonucleic acid, which also

*) (fractions C 1–3 according to Scopes' nomenclature, *Scopes*, 1963)


A
B
SERUM
alb.
α, α,β γ
α, α, β
DIF

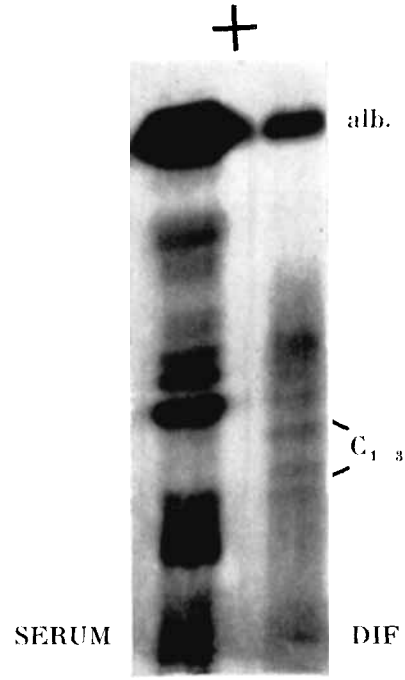


Fig. 2. Starch gel electrophoresis of proteins of serum and DIF from swine.

involving the use of physiological saline. In this application of elution for the isolation of an interstitial fluid there are also several possible sources of error. The volume of the fluid obtained by elution must be calculated from the volume of the

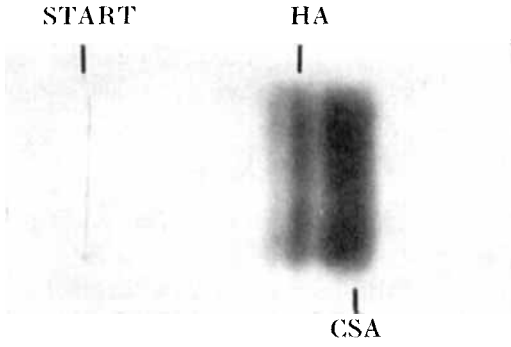


Fig. 3. Electrophoretic pattern of acid mucopolysaccharides in DIF after hydrolysis with papain.

dentine canals or from the content of free water in the dentine (*Spreter v. Kreudenstein & Stüben,* 1956), which makes volume determinations laborious and also inaccurate. It is also obvious that during the rather long elution period some material dissolves from the solid dentine in the eluate. The high concentrations of electrolytes in the eluate of dentine may be considered as evidence of this. Thus the sodium content of dentine eluate has been reported to exceed 300 mEq/l (more than 700 mg/100 ml, *Spreter v. Kreudenstein,* 1963). According to *Spectrum* (1956) the sodium concentration of swine serum has the range of 140—160 mEq/l.

In the isolation method presented in which centrifugal force is employed, the absolute volume of the fluid obtained can be measured directly. It is obvious that all the fluid in the interstitial spaces is not isolated by these means. However, the samples may be regarded as representative of the average composition of DIF. The contamination of the isolated fluid by substances eluted from solid dentine structures can be avoided, at least as far as electrolytes are concerned. The sodium concentration was 149.6 mEq/l in DIF and 155.0 mEq/l in the serum of the same animal.

Our values for the proteins of the DIF differ from those of *Spreter v. Kreudenstein* (1959), who reported values of 0.125 g/100 ml for total proteins and 20—30 mg/100 ml for non-protein nitrogen. The total nitrogen content of DIF isolated by our method was 246.4 mg/100 ml. From this value are subtracted the amount of non-protein nitrogen (28.7 mg/100 ml) and the calculated amount of protein and haemoglobin nitrogen in the contaminating blood (38.7 mg/100 ml). We then obtain a total protein content of 1.10 g/100 ml. As judged from the high hydroxyproline content, part of the protein must be soluble collagen or hydroxyproline-containing peptides. The amount of protein corresponding to the hydroxyproline content (13.69 mg/100 ml) is 0.10 g/100 ml when calculated as collagen, using a factor of 7.3.

Direct measurement of the protein content of interstitial fluids has been inaccurate because of difficulties in isolation. The values reported vary from less than 1 per cent to 2—3 per cent,

according to isolation method and tissue (*Landis*, 1946; *Pitts*, 1963).

There are distinct qualitative differences between DIF and serum. The former contains quite large amounts of substances which are without doubt derived from connective tissue, e.g. the hydroxyproline-containing material. Moreover, its relative carbohydrate content (according to hexosamine analysis) is much higher than that of serum. Only a small fraction of the carbohydrate material consists of acid mucopolysaccharides (Table 1).

On the basis of the qualitative differences between the electrophoretic glycoprotein patterns of DIF and serum (Fig. 1 B) we can assume that the bulk of the PAS-positive, hexosamine-containing material in DIF is not derived from serum. In the DIF almost all PAS-positive proteins move like β-globulins, but in the serum all the conventional glycoprotein fractions can be seen, the α_2-globulin band being the strongest. This carbohydrate material, which does not originate from the serum, obviously consists of the glycoproteins and neutral heteropolysaccharides of connective tissue (e.g. *Dische et al.*, 1958).

The starch gel protein electropherogram (Fig. 2) likewise shows differences between DIF and serum. For example, the bands after albumin in the serum pherogram (α_2-globulin?) are lacking and some of the bands in the haptoglobin region are stronger in DIF. (One must take into account that the DIF contained a small amount of haemoglobin, which may somewhat confuse the picture.) *Fricke* (1962), using immunoelectrophoresis, found that some of the components of serum proteins could not be demonstrated in the interstitial spaces of various connective tissues. This is consistent with the observations of *Rejnek & Bednarik* (1960), who studied the starch gel electrophoretic patterns of interstitial fluids isolated from parenchymatous organs. These differences between the protein patterns of interstitial fluids and serum have been interpreted by assuming some kind of special barrier system between the vascular and extravascular spaces (*Pappenheimer*, 1953; *Rejnek & Bednarik*, 1960; *Bazin & Delaunay*, 1961), or by differences in the affinities of the components of connective tissue for different serum proteins (*Fricke*, 1962).

Our results confirm the view that the interstitial fluid cannot

be considered to be a mere ultrafiltrate of blood. Besides serum proteins the interstitial fluid isolated from dentine contains materials which are characteristic of the amorphous ground substance of connective tissue (e.g. *Gibian,* 1959; *Bazin & Delaunay,* 1961). It is known that the colloidal state of ground substance varies from sol to gel. Obviously DIF should not be classified as a distinct kind of tissue fluid, but has to be regarded as the soluble ground substance of dentine.

SUMMARY

A method is reported for isolation of the interstitial fluid of dentine by centrifugal force, and data from chemical and electrophoretic studies on its composition are presented. In addition to components derived from blood, the isolated fluid consists of soluble protein and carbohydrate material which obviously originate from connective tissue.

Acknowledgments

The authors would like to thank Prof. *A. Scheinin,* who proposed this investigation, for helpful discussions and criticism during the work. Our thanks are also due to Messrs. *Lounais-Suomen Osuusteurastamo,* Turku, for the specimens of teeth and to Mrs. *M. Potila,* who helped to carry out the chemical determinations. The samples of acid mucopolysaccharides used as reference in electrophoresis were gifts from Prof. *K. Meyer,* New York, N.Y. and Dr. *M. B. Mathews,* Chicago, Illinois, U.S.A.

RÉSUMÉ

ÉTUDES SUR L'ISOLEMENT ET LA COMPOSITION DU FLUIDE INTERSTITIEL DE LA DENTINE DU PORC

Les auteurs rendent compte d'une méthode destinée à isoler le fluide interstitiel de la dentine par la force centrifuge, et présentent les renseignements fournis par des études chimiques et électrophorétiques sur sa composition. Outre des éléments dérivés du sang, le fluide isolé consistait en protéine soluble et en hydrates de carbone provenant manifestement du tissu conjonctif.

ZUSAMMENFASSUNG

UNTERSUCHUNGEN ÜBER ISOLIERUNG UND ZUSAMMENSETZUNG VON INTERSTITIELLER DENTINFLÜSSIGKEIT DES SCHWEINES

Eine Methode zur Isolation der interstitiellen Dentinflüssigkeit durch die Zentrifugalkraft wird beschrieben, und Resultate von chemischen und elektrophoretischen Erforschungen über die Zusammensetzung der erwähnten Flüssigkeit werden dargestellt. Die isolierte Flüssigkeit besteht, ausser den von der Blut herrührenden Bestandteilen, aus irgend einem löslichen Protein und einem Kohlenhydratmaterial, die offensichtlich von der Bindegewebe herrühren.

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