Electrophoresis Studies of Human Synovial Mucin

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A characteristic property of synovial fluids is the formation of a mucin clot when a weak acid is added. The physical character of the clot may be of some aid in the diagnosis of rheumatoid arthritis (Ropes, Bennett, Cobb, Jacox & Jesser, 1957; Ropes & Bauer, 1953). The clot has also been used as a preliminary step in the isolation of hyaluronic acid (Meyer, Smyth & Dawson, 1939) and its hexosamine content as a measure of the hyaluronic acid (Ogston & Stanier, 1950; Hamerman & Schuster, 1958).

Previous electrophoretic studies of synovial mucin have indicated that the clot is composed of hyaluronic acid and protein (horse mucin; Blix, 1940); hyaluronic acid, albumin and γ -globulin (horse and human mucin; Hesselvik, 1940); and hyaluronic acid or complex, albumin and other proteins (Ogston & Stanier, 1952; Ropes, Robertson, Rossmeisl, Peabody & Bauer, 1947). Chemical studies had shown hyaluronic acid and 'globulin' as components (Meyer *et al.* 1939), whereas ultracentrifuge analyses showed the presence of a hyaluronic acid complex and albumin (cattle mucins; Ogston & Stanier, 1950, 1952).

The work described in this paper is a part of a basic study of the composition and significance of human synovial mucin. The composition as indicated by electrophoretic analysis is the principal subject of this paper.

METHODS AND MATERIALS

Sources. Normal and post-mortem synovial fluids comprised the 'normal' group (Platt, Pigman, Holley & Patton, 1956). All were pooled fluids. The post-mortem fluids were obtained from the knee joint at hospital autopsy a few hours after the death of the patient. The normal fluid was a pooled sample obtained from the knee joints of about ten young male volunteers (average age 20 years).

The pathological synovial fluids were divided into two groups: the rheumatoid-arthritis group and a miscellaneous group containing the osteoarthritis and Reiter's-

syndrome fluids. No significance was ascribed to the clinical state of the disease.

Preparation of clot. For the preparation of the clot, an equal volume of 5% acetic acid was added to the synovial fluid (pH 4.0). The solution was centrifuged, and the supernatant was decanted and saved. The clot was washed three times with water and the washings were discarded. The clot was stirred in 1% sodium carbonate solution, equal in volume to that of the original synovial fluid sample. Upon stirring, the clot dissolved. The solutions were then prepared for electrophoretic analysis. In several instances, the clots were reprecipitated and redissolved, once as many as five times. All procedures were carried out as rapidly as possible at 4°.

Electrophoretic analysis. For the electrophoretic analyses, the original synovial fluid was diluted with 2 vol. of buffer. Buffer (1 vol.) was added to 2 vol. of the supernatant solution remaining after separation of the clot. Buffer (2 vol.) was added to the dissolved clot solution. The solutions were then dialysed at 4° in Visking cellulose casing against 500 ml. of buffer for 48 hr. The buffer used was sodium diethylbarbiturate (pH 8.6, *I* 0.1).

The Perkin-Elmer Tiselius-type of electrophoresis apparatus with a 6 ml. cell was used at 0.5°, with a constant current of 9 mA and a time ranging from 13 800 to 20 400 sec. The ascending patterns were used for all mobility determinations, for reasons previously described (Platt *et al.* 1956; Pigman, Patton & Platt, 1957). The concentrations of the various electrophoretic components were calculated from tracings of the electrophoretic patterns enlarged from three to four times. The area for each individual boundary was cut out and weighed and reported in Table 2 as percentage of total refractive-index increment.

Viscosity. Viscosity measurements were made at 30° with Cannon-Manning semi-microviscometers (Cannon Instrument Co., State College, Box 812, Penn., U.S.A.). Water had a flow time of 90 sec. for 0.2 ml. for the usual viscometer; for very viscous fluids, the viscometer had a flow time of 3.7 sec. for 0.23 ml. of water. The measurements were carried out on the diluted fluids as used for electrophoresis. For these measurements, each synovial fluid was diluted to three times its original volume with buffer and dialysed against buffer (pH 8.6, *I* 0.1). Clots were dissolved as indicated above and the solutions were diluted and dialysed as described for the original fluids.

Ultracentrifugal analysis. Ultracentrifugal analyses were carried out in the Spinco model E analytical ultracentrifuge at 59 780 rev./min. (259 700 *g*) with the 12 mm. standard analytical cell and the same solutions as for electrophoresis and viscosity measurements.

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RESULTS

Results for the electrophoretic analyses of the mucin clots obtained from human synovial fluids are shown in Table 1. On the basis of the known electrophoretic mobilities of the components of human sera and synovial fluids (Platt *et al.* 1956; Pigman *et al.* 1957), the clot components were identified as indicated in Table 1. All clots contained hyaluronic acid and albumin. α_1 -, α_2 - and β -Globulins could be detected in some of the clots. The γ -globulin component of serum could not be demonstrated in the clots, and none of them contained more than two identifiable globulin components. The peaks which appeared to be questionable or diffuse are indicated in Table 1 by asterisks.

The π -peak, previously shown to occur in many normal and post-mortem synovial fluids (Platt *et al.* 1956; Pigman *et al.* 1957), was detected in the clot obtained from one of the post-mortem fluids and can be seen in Fig. 1 (c), and in the clot obtained from the pooled normal fluid. The π -component was not found in any of the clots obtained from the pathological fluids. A typical pattern of the clot obtained from the pathological fluids is shown in Fig. 1 (f).

All of the hyaluronic acid appeared to be precipitated with protein to form the mucin clot, since, in the supernatant, hyaluronic acid could not be detected electrophoretically, (Fig. 1b). Under similar conditions, Hamerman & Schuster (1958) reported that all hyaluronic acid was found in the clot.

The clots from post-mortem fluids seemed to be composed almost exclusively of the hyaluronic acid component and albumin (Fig. 1c). Globulins, when apparently present, were nearly always questionable. The pooled normal fluid, however, had definite signs of α - and β -globulins. The clots from arthritic fluids showed more regularly the presence of globulins, especially α -globulins.

The amount of clot was always much less for arthritic fluids than for normal and post-mortem fluids. Data on the weights of clots will be given in subsequent papers. Reprecipitation of several clots did not make any noticeable change in their composition. A comparison of the electrophoretic pattern of the original clot (from an arthritic synovial fluid) with that of a twice-reprecipitated and redissolved clot is shown in Fig. 2. Similar results were obtained after five reprecipitations. The final clots appeared to be very similar to the

Table 1. *Absolute electrophoretic mobilities in the ascending limb for the mucin clot obtained from human synovial fluid*

Values of mobility are given in $-10^5 \times (\text{cm}^2 \text{v}^{-1} \text{sec}^{-1})$. PM, Post-mortem; RA, rheumatoid arthritis; OA, osteoarthritis; RS, Reiter's syndrome; N, normal. (For details see text.) The last column gives the ratio of viscosity of the clot to that of the fluid from which it was derived under similar conditions of dilution and ionic strength.

Type of synovial fluid	Hyaluronic acid	π^*	Albumin	Globulin			Viscosity (centipoises)	Viscosity of original fluid
				α_1	α_2	β		
PM	8.6	—	6.4	—	—	—	1.4	0.47
PM	8.8	—	6.6	—	—	—	3.3	0.62
PM	8.9	—	6.6	—	5.0†	—	2.9	0.18
PM	9.6	—	7.4	—	—	—	1.5	—
PM	8.3	7.8	6.5	—	5.0†	3.9†	31	0.58
PM	8.6	—	6.4	—	5.0†	3.9†	6.1	0.10
N	8.9	8.4	6.8	5.6	—	3.9	23	0.18
Average	8.8	8.1	6.7	5.6	5.0	3.9	9.9	—
Range	8.3–9.6	7.8–8.4	6.4–7.4	—	—	—	—	—
RA	9.2	—	6.7	5.4	—	4.0	—	—
RA	9.1	—	6.4	5.8†	4.9†	—	1.7	0.77
RA	9.2	—	6.7	5.2	4.5	—	2.0	0.91
RA	9.0	—	6.7	—	4.5	—	2.3	—
RA	8.8	—	6.5	5.1	—	—	1.7	0.40
RA	8.6	—	6.5	—	4.6	—	—	—
RA	8.7	—	6.4	—	4.6†	3.5	2.0	—
RA	8.7	—	6.6	—	4.8†	3.7†	2.1	—
RA	9.0	—	6.5	5.6	4.8	—	1.7	—
Average	8.9	—	6.6	5.4	4.7	3.7	1.9	—
Range	8.6–9.2	—	6.4–6.7	5.1–5.8	4.5–4.9	3.5–4.0	—	—
OA	8.8	—	6.5	5.1	—	—	1.8	—
OA	8.9	—	6.6	—	4.3	—	1.8	—
RS	8.8	—	6.7	5.2†	—	—	2.6	—

* The π component appears in many normal and post-mortem fluids (see text).

† These peaks appeared diffuse or were questionable.

original, and the composition remained fairly constant, although the absolute amounts had decreased a little as a result of repeated treatment. The apparent shift of albumin to α_1 -globulin, shown in Table 2, probably arises from experimental error and the poor resolution of the α_1 -peak.

Several electrophoretic patterns were available in which the hyaluronic acid component was sufficiently sharp to allow measurements of the relative areas (and concentrations). The results are shown in Table 2. In the original fluid, the ratio of protein to hyaluronic acid varied widely. However, in the clot obtained from normal, post-mortem and rheumatoid-arthritic synovial fluids, the hyal-

uronic acid component appeared to account for 12–15 % of the total area of the clot pattern. When corrected for the differences in the specific refractive-index increment of hyaluronic acid and protein [Curtain (1955) gives these as 0.00182 for protein and 0.00144 for hyaluronic acid] this range is raised to 15–18 %.

In the ultracentrifugal patterns of the normal and post-mortem clots, two components corresponding in sedimentation behaviour to hyaluronic acid and albumin could be detected. Ogston & Stanier (1950) found these components in cattle synovial mucins. The pathological clots contained small amounts of globulin in addition to hyaluronic acid and albumin. The very sharp peak of the hyaluronic acid component appeared to be virtually unchanged in the clot. Hyaluronic acid could not be detected in the supernatant solutions.

The viscosity of the clot was one-tenth to three-fifths of the viscosity of the original synovial fluids in the 'normal' group under comparable conditions of dilution (Table 1). In the pathological group, the viscosity of the dissolved mucin-clot solutions

Fig. 1. Ascending electrophoretic patterns of post-mortem (PM) and pathological human synovial fluids, the supernatant and dissolved mucin clot. (a) Original PM fluid; (b) PM supernatant; (c) PM mucin clot; (d) original rheumatoid-arthritic (RA) fluid; (e) RA supernatant; (f) RA mucin clot. Veronal buffer, pH 8.6, *I* 0.1; current, 9 mA; time, 230–340 min. HA, Hyaluronic acid.

Fig. 2. Ascending electrophoretic patterns of (a) the dissolved mucin clot first obtained from rheumatoid-arthritic synovial fluid, and (b) the clot obtained after it was reprecipitated and redissolved twice. Veronal buffer, pH 8.6, *I* 0.1; current, 9 mA; time, 225 min. HA, Hyaluronic acid.

Table 2. Relative refractive increments of components in human synovial fluid and in the mucin clot as measured directly from the electrophoretic patterns

Fluid	Refractive increment (% of total)						
	Hyaluronic acid	π	Albumin	Globulin			
				α_1	α_2	β	γ
Original post-mortem fluid	7	3	62	5	5	9	10
Clot (post-mortem)	15	—	75	—	10	—	—
Original fluid (normal)	11	16	44	4	—	16	9
Clot (normal)	15	4	73	6	—	1	—
Original fluid (RA)	2	—	58	1	1	8	30
Clot (RA)	12	—	65	14	9	—	—
Clot (RA), redissolved and repptd. twice	14	—	54	21	11	—	—

ranged from two-fifths to nine-tenths of the value of the original fluid. The supernatant fluids had viscosities corresponding to those of similarly diluted sera.

Since the possibility existed that the treatment with alkali could degrade the hyaluronic acid, the dissolved mucin-clot solutions were kept at 30° and pH 10 for 48 hr. The final viscosity of 16 centipoises was only slightly altered from the original viscosity of 18 centipoises under the same conditions.

DISCUSSION

The electrophoretic analyses of human synovial mucin show clearly that the principal components are hyaluronic acid (or complex) and albumin. As indicated by the patterns and the viscosity of the supernatant fluids, all hyaluronic acid appears in the mucin. These results agree with recent chemical analyses (Hamerman & Schuster, 1958).

Minor amounts of α - or β -globulins may be present in the clots, especially in those from patients with rheumatoid arthritis. The appearance of these globulins may be related to the concentration of all proteins greater in arthritic synovial fluids as compared with normal or post-mortem fluids (Platt *et al.* 1956).

Of the previously described compositions for synovial mucins, these results agree most closely with those of Ropes *et al.* (1947) and of Ogston & Stanier (1950, 1952) for cattle synovial mucin. Ogston & Stanier (1950), however, considered that the hyaluronic acid is present as a 'complex' with protein. This complex is reported to consist of about 75 % of hyaluronic acid and 25 % of protein, mainly α_2 -globulin according to Curtin (1955). The complex comprises 45 % of the cattle synovial mucin. The hyaluronic acid would thus compose 34 % of the mucin.

Ropes *et al.* (1947) give the hyaluronic acid as 15 % of cattle mucin, determined by hexosamine analysis. The data given by Hamerman & Schuster (1958) for the nitrogen and hexosamine contents of clots from human post-mortem fluids give an average content of hyaluronic acid of 20.5 % when the hexosamine content of hyaluronic acid is taken as 47.2 %. These values are in close agreement with those for human synovial mucin, 15–18 %, as reported in the present work. Ogston & Stanier's (1950) values, however, are about twice as great, but these workers (1952) point out that the extent of washing may influence the composition.

No evidence for a stable hyaluronic acid-protein complex was obtained in this work. Under the conditions of electrophoresis, the fast component clearly does not comprise 45 % of the total clot. Its mobility also corresponds to those for hyaluronic

acids isolated from various sources. The problem is under further study, and the results will be reported later.

Some of the human mucins showed π -peaks in the electrophoretic patterns. However, in several instances this peak was not present in the pattern for the mucin, although shown in that for the original fluid. The significance of this is not known.

The relative constant percentage of apparent hyaluronic acid in three clots from normal subjects, post-mortem and rheumatoid-arthritis patients may be a coincidence, but this finding agrees with the claim of Meyer, Palmer & Smyth (1937) that a definite combining ratio exists. The lack of marked change upon reprecipitation is also in agreement with a fixed ratio.

SUMMARY

1. Human synovial mucin was prepared from normal, post-mortem and pathological fluids, including many from patients with rheumatoid arthritis.

2. Electrophoretic studies at pH 8.6 show that the main constituents are albumin and a fast-moving component. The fast-moving component seems to be hyaluronic acid but might be the complex described by Ogston & Stanier (1950).

3. Particularly in clots from arthritic fluids, α - and β -globulins may be present, though in small amounts. No evidence was obtained for γ -globulins.

4. Analysis of the electrophoretic patterns indicated the presence of 15–18 % of hyaluronic acid in a few clots from normal and pathological fluids. This apparent constancy and the fact that only slight changes were caused by reprecipitation suggest a constant combining ratio of hyaluronic acid and protein.

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The Respiration of Cardiovascular Tissues of the Developing Chick and Adult Fowl

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Little information is available on the oxygen uptake of cardiac tissue, one probable reason for this being the technical difficulty of making accurate observations on such tissue owing to the rapidity with which it deteriorates after removal from the experimental animal (Bernheim & Bernheim, 1944; Webb, Saunders & Thienes, 1949*a*). Values have been reported for the mouse heart by Pearce (1936), for the rat heart by Bertalanffy & Pirozynski (1951), Webb *et al.* (1949*a*) and Nowy & Helmreich (1950), but only a few observations have been made on the chick-embryo heart (Warburg & Kubowitz, 1927).

The present study mainly deals with the oxygen uptake of cardiac tissue in the chicken (*Gallus domesticus*) during incubation, after hatching and in adult cockerels. The results are compared with histological changes observed in the heart during the various stages of development. In addition, further observations were made on the aorta to supplement the previously reported work of Henderson & MacDougall (1956).

MATERIALS AND METHODS

Determination of oxygen uptake

The differential microrespirometer described by Cruickshank (1954) was used for determining the oxygen uptake of tissue slices. Chick embryos ranging in age from 5 to 22 days of incubation, chickens aged 1–16 days after hatching and mature cockerels provided the tissues. In addition to the incubation age, the weight of each embryo minus its yolk sac was recorded. Observations on the external form of the embryo as described by Hamilton (1952) for determining the stage of development were made, but were not so useful for the present purpose as age and weight.

The method was essentially the same as that used by Henderson & MacDougall (1956) in the study of the respiration of chick-embryo arteries. Immediately after removal from the bird, the heart was placed in ice-cold Krebs-

Ringer phosphate and cut by free-hand slicing into 0.5 mm. thick fragments. Preliminary experiments showed that slices which were much thicker or thinner than 0.5 mm. gave less than optimum Q_{O_2} values. The speed of transfer of the tissue from the animal, the temperature of the Krebs-Ringer phosphate and the thickness of the slices proved to be factors more critical than in the previous work on arteries.

The medium was that used by Cruickshank (1954), but without streptomycin since prolonged observation was not required. It consisted of 0.5 ml. of horse serum, 0.3 ml. of Krebs-Ringer phosphate and 0.1 ml. of 5% (w/v) glucose solution. The Krebs-Ringer phosphate solution was composed of (as parts by vol.) 0.154 M-NaCl (100); 0.154 M-KCl (4); 0.11 M-CaCl₂ (3); 0.154 M-MgSO₄·7H₂O (1). To this was added phosphate buffer (21 parts) prepared by mixing 40 ml. of 0.25 M-Na₂HPO₄ with 2 ml. of N-HCl and diluting this mixture to 100 ml. The tissue slices were placed on lens paper and held floating on the surface of the medium on a platform of tantalum gauze. The gas phase was air, and carbon dioxide was absorbed by filter paper soaked in KOH (2%, w/v). It is possible that higher values for oxygen uptake might have been obtained with oxygen as the gas phase, but this was not used as it was desired to compare the results of the present investigation with those of Henderson & MacDougall (1956). After thermal equilibration for $\frac{1}{2}$ hr. in a water bath at 38°, readings were taken every 10 min. for periods of up to 2 hr., during which time the oxygen uptake was found to be linear for at least the first hour in all cases. After the tissue slices had been dried to constant weight at 100° the uptake of oxygen was calculated as $Q_{O_2} = \mu\text{l. of } O_2/\text{mg. dry wt. of tissue/hr.}$

Histological methods

Portions of the atrium, apex of the ventricle and aorta adjacent to those used for the respiration studies were examined by a variety of histological methods. Immediately after the death of the animal the tissues were placed in one or more of the following fixatives: Ringer-formol, formol corrosive and Zenker's fluid. After identical processing, paraffin sections (10 μ) were stained by standard methods for connective tissues. To demonstrate collagenous fibres Van Gieson's stain, Mallory's aniline blue and Masson's trichrome (light-green) stain were used. For