

NONDIALYZABLE HEXOSE OF HUMAN SYNOVIAL FLUID * †

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(Submitted for publication November 24, 1959; accepted January 29, 1960)

Studies that show differences in the components of synovial fluids in osteoarthritis and rheumatoid arthritis may be helpful diagnostically. Synovial fluids from joints involved by rheumatoid arthritis contain a high concentration of protein, and electrophoretic studies reveal increased α_2 -globulins (1, 2). Since this protein fraction contains the highest concentration of hexose among the electrophoretically separated proteins of serum (3), determination of nondialyzable hexose concentration in synovial fluid was undertaken. In synovial fluid from osteoarthritic joints the nondialyzable hexose concentration, determined with the anthrone reagent, was similar to or lower than the normal level, while in synovial fluid from joints involved by rheumatoid arthritis it was elevated.

The first step in this study was to identify the carbohydrate components of dialyzed synovial fluid that contributed to the anthrone reaction. Synovial fluid was dialyzed and then lyophilized. The lyophilized solids were hydrolyzed in sulfuric acid, and the carbohydrates in the hydrolysate were separated by a combination of zone electrophoresis in borate buffer and paper chromatography. Galactose, mannose, small amounts of fucose and glucose, and hyalobiuronic acid were found.

MATERIALS AND METHODS

I. Identification of carbohydrate components

Synovial fluid from normal subjects. Synovial fluid was aspirated from apparently normal knee joints of deceased subjects. Fluids from 8 to 10 normal knees were pooled to provide about 10 ml. Three different

* Part of this work was presented before the Second Pan-American Congress on Rheumatic Diseases, June 1959.

† Supported by Graduate Training Grant 2A-5082 from the National Institute of Arthritis and Metabolic Diseases, and by a grant from the N. Y. State Chapter, Arthritis and Rheumatism Foundation.

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pools of normal synovial fluid were collected. The pooled fluids were diluted with an equal volume of a buffer (0.03 M NaHCO₃ and 0.15 M NaCl, pH 8.1), dialyzed against 500 ml of distilled water (changed twice daily) for 48 hours at 2° C, and lyophilized.

*Synovial fluid from patients with primary osteoarthritis or rheumatoid arthritis.*¹ About 10 ml of synovial fluid was obtained from the involved knee joint of 1 patient with primary osteoarthritis and from 2 patients with rheumatoid arthritis. These fluids were treated in the same manner as the pooled normal synovial fluids.

Normal serum. The total protein-bound hexose of pooled normal serum was precipitated with ethanol according to the method of Winzler (4), and dried in a vacuum over P₂O₅.

Hydrolysis. Lyophilized solids (80 mg) of pooled normal synovial fluid were hydrolyzed in 1 ml of 2 N H₂SO₄ in a sealed tube for 2 hours at 100° C. The hydrolysate was adjusted to pH 5.0 with Ba(OH)₂, and the insoluble BaSO₄ removed by centrifugation. Lyophilized solids of osteoarthritic synovial fluid (100 mg), rheumatoid synovial fluid (100 mg), and the dried precipitate of normal serum (100 mg) were similarly hydrolyzed and neutralized.

Zone electrophoresis. The technique employed was modified from that of Müller-Eberhard and Kunkel (5). Blocks (4 × 13 × ¼ inches) of polyvinyl chloride (Geon Resin no. 435, obtained from B. F. Goodrich) were used. The hydrolysate of normal synovial fluid was applied to the block in a slit (½ × ⅛ × ¼ inches) 3 inches from the cathodal end of the block. A mixture of authentic glucose, galactose and mannose was applied to a slit in the block 2 inches lateral to the hydrolysate. Electrophoresis was carried out for 9 hours in borate buffer (0.05 M Na₂B₄O₇ · 10 H₂O and 0.15 M H₃BO₃, pH 8.5) at a voltage gradient of 12.5 volts per cm at 2° C. The block was blotted dry and divided longitudinally into two equal parts. Each part was cut into ¼ inch segments starting at the anodal end. Each segment was placed in a 15 ml sintered glass funnel (medium porosity), and elution was performed with 3 additions (2.5 ml each) of distilled water by applying air pressure to the funnel. The final volume of each eluate was made up to 8 ml. After analysis for hexose, the eluates of some segments

¹ For simplicity, synovial fluids obtained from involved joints of patients with osteoarthritis or rheumatoid arthritis will be termed osteoarthritic synovial fluid and rheumatoid synovial fluid, respectively.

were pooled to obtain a larger quantity of the isolated carbohydrates.

Electrophoresis of the hydrolysates of osteoarthritic synovial fluid, rheumatoid synovial fluid, and normal serum was similarly performed.

Removal of sodium borate. It was necessary to remove sodium borate from the eluates prior to chromatography. The eluates containing *hexoses* were passed through a column (5 × 0.9 cm) of Amberlite 1R-120 (H⁺ form). The effluent was evaporated to dryness in a vacuum, methanol (3 additions of 5 ml each) added, and the methyl borate removed by vacuum distillation at 30° C (6). The eluates containing *hyalobiuronic acid* were passed through a column (3 × 0.9 cm) of equal parts (by weight) of carbon and celite (7). The sodium borate was washed from the column with distilled water (3 additions of 10 ml each). The hyalobiuronic acid was then eluted from the column with 10 ml of 30 per cent (vol/vol) aqueous ethanol.

Chromatography. Ascending paper chromatography was performed using Whatman no. 1 paper at room temperature for 16 hours. Hexoses were separated in isopropyl alcohol-water (8:2) (8), and the chromatograms were stained with aniline hydrogen phthalate. Eluates containing hyalobiuronic acid were chromatographed in butanol-acetic acid-water (6:2:2), and the chromatograms were stained with either aniline hydrogen phthalate or *p*-dimethylaminobenzaldehyde in HCl (8).

Analytical methods. All the eluates were analyzed for hexoses by an anthrone method (9). Anthrone reagent was prepared by adding 100 ml of C.P. H₂SO₄ to 0.2 g of anthrone. Five ml of anthrone reagent was added to Pyrex test tubes (25 × 150 mm) in water at 10° C. An aliquot (2.5 ml) of each eluate was layered on top of the anthrone reagent. A reagent blank (2.5 ml of the NaHCO₃-NaCl buffer) and the hexose standard (1 ml containing 50 μg per ml of galactose and 50 μg per ml of mannose plus 1.5 ml of buffer) were similarly prepared. The tubes were covered with glass marbles, shaken, removed from the water, and allowed to stand at room temperature for 5 minutes. The tubes were immersed in water at 100° C for exactly 6 minutes, and then cooled in water at 10° C for 5 minutes. Optical density was determined in a Beckman DU spectrophotometer at 625 mμ.

Some of the eluates were also analyzed for hexosamine (10) and hexuronic acid (11). Lyophilized nondialyzable solids of synovial fluids were analyzed for hexose, hexosamine and nitrogen (12).

11. Determination of nondialyzable hexose concentration

Synovial fluid was obtained from two groups of subjects with apparently normal knee joints. The first group consisted of 22 deceased subjects of varying ages who had no evidence of acute illness. The second group, was composed of living subjects in coma from an acute cerebral vascular accident. The methods of aspiration and preservation of the fluid are described elsewhere (10). Synovial fluid was also obtained from *involved*

knee joints of patients with active rheumatoid arthritis or primary osteoarthritis. The diagnosis of definite rheumatoid arthritis fulfilled the criteria of the American Rheumatism Association (13). The diagnosis of primary osteoarthritis was established by history, physical examination, X-ray changes, normal erythrocyte sedimentation rate, and negative latex fixation test. Serum was obtained from the living subjects at the time synovial fluid was aspirated.

Dialysis. To a weighed aliquot (0.2 g) of normal or osteoarthritic synovial fluid was added 2.5 ml of a buffer (0.03 M NaHCO₃, 0.15 M NaCl, pH 8.1). To rheumatoid synovial fluid (0.15 g) 3 ml of buffer was added. Each diluted fluid was mixed, added to cellulose casing (Visking, 8/32 inch) and dialyzed against 500 ml of buffer changed twice daily for two days at 2° C. To weighed aliquots (0.25 g) of serum from normal subjects or patients with osteoarthritis was added 3 ml of buffer. A smaller aliquot (0.15 g) of serum from patients with rheumatoid arthritis was similarly diluted with buffer. The diluted sera were then dialyzed against buffer.

Alcohol precipitation. In a few synovial fluids and sera, the values for nondialyzable hexose were compared with protein-bound hexose precipitated by alcohol. Weighed aliquots similar to those described above were taken. Normal synovial fluid was diluted 1:5 with tap water; rheumatoid synovial fluid was diluted 1:1; serum was not diluted. To the diluted fluids and sera 10 ml of cold absolute ethanol was added with constant stirring. After standing for 30 minutes at 2° C, the tubes were centrifuged, the precipitate washed once in ethanol, and then dissolved in 2 or 3 ml of buffer.

Hexose analysis. To 5 ml of anthrone reagent in Pyrex test tubes immersed in water (10° C), 0.5 ml buffer and 2 ml² of diluted dialyzed synovial fluid were carefully added down the side of the tube. For analysis of serum, 1.5 ml of buffer and 1 ml of diluted, dialyzed serum were used. A hexose standard (1 ml containing 50 μg per ml of galactose and 50 μg per ml of mannose plus 1.5 ml of buffer) and a reagent blank (2.5 ml of buffer) were similarly prepared. The determination was carried out as described above.

Protein determination. A biuret method was used (14). When the total volume of synovial fluid was less than 0.3 g a modified Folin method (15) was employed to permit analysis of protein in 0.05 g of synovial fluid. Normal serum, whose nitrogen was determined by a micro-Kjeldahl method (12), was used as a standard. Values for protein obtained by the biuret and Folin method on the same synovial fluids agreed within 5 per cent.

Anthrone reaction of isolated hyaluronidase-digested hyaluronate. Synovial fluid was digested with hyaluronidase, and the hyaluronate fragments isolated by zone electrophoresis in a phosphate buffer (0.05 M, pH 6.5) on blocks of polyvinyl chloride (16). The molar ratio

² The density of diluted synovial fluid is 1, and grams and milliliters may be used interchangeably.

TABLE I
Analyses of nondialyzable solids of human synovial fluid

	Weight of solids (mg/g) of synovial fluid	Hexose	Hexosamine	Nitrogen
		%	%	%
Normal	19.9	1.8	5.1	12.2
Normal	17.7	1.8	5.5	
Normal	17.0	2.0		
Osteoarthritis	39.8	1.6	3.1	12.1
Rheumatoid arthritis	64.4	1.3	2.0	10.2
Rheumatoid arthritis	43.8	1.5	2.8	

of hexuronic acid to hexosamine of the isolated hyaluronate fragments was 1. No protein was detected by the Folin method (15) and chromatography after acid hydrolysis revealed hexosamine but no amino acids. The anthrone reaction of an aliquot of this material containing 400 μ g of hexuronic acid per ml was determined.

RESULTS

I. Identification of carbohydrate components

Analytical data. The hexose, hexosamine and nitrogen content of the nondialyzable solids of

normal, osteoarthritic and rheumatoid synovial fluid are shown in Table I.

Hydrolysate of normal synovial fluid. Anthrone-reacting carbohydrates in the acid hydrolysate of nondialyzable solids of normal fluid were separated into five zones by electrophoresis (Figure 1). Three of these five zones (Zones I, II and IV) had mobilities nearly identical with those of authentic glucose, galactose and mannose. The carbohydrates in the pooled eluates of these zones were studied by chromatography, and glucose,

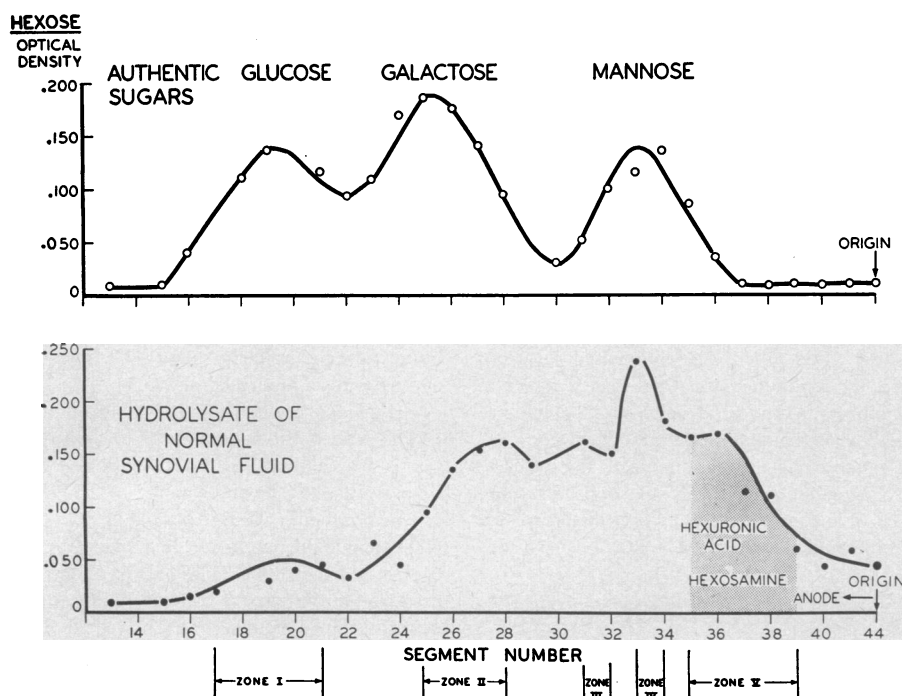


FIG. 1. ZONE ELECTROPHORESIS OF AN ACID HYDROLYSATE OF NONDIALYZABLE SOLIDS OF NORMAL HUMAN SYNOVIAL FLUID, AND OF AUTHENTIC HEXOSES IN BORATE BUFFER, pH 8.5. Eluates were analyzed for carbohydrate with the anthrone reagent and the optical density was plotted for each segment. Eluates within each of five zones were then pooled for chromatographic studies.

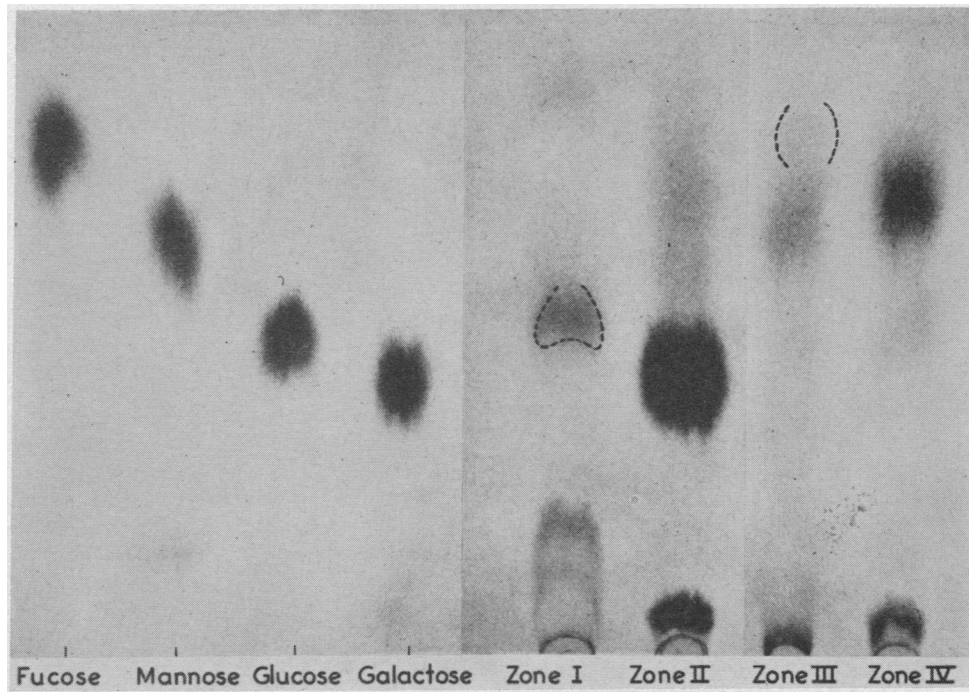


FIG. 2. ASCENDING PAPER CHROMATOGRAM OF HEXOSES IN ZONES I THROUGH IV OBTAINED AFTER ZONE ELECTROPHORESIS, AND OF AUTHENTIC HEXOSES. Isopropyl alcohol-water solvent (8:2); aniline hydrogen phthalate stain. Zone I, glucose; Zone II, galactose; Zone III, traces of fucose and mannose; Zone IV, mannose.

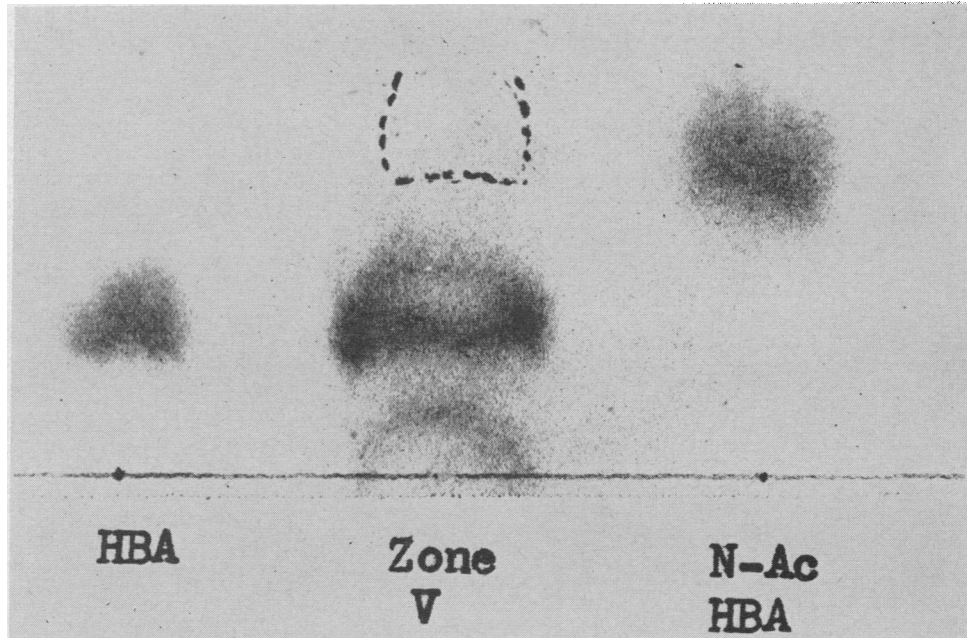


FIG. 3. ASCENDING PAPER CHROMATOGRAM OF HYALURONATE FRAGMENTS IN ZONE V. Butanol-acetic acid-water solvent (6:2:2); *p*-dimethyl aminobenzaldehyde in HCl stain. HBA, hyalobiuronic acid (red); N-AcHBA, N-acetyl hyalobiuronic acid (purple). In Zone V the faint purple stain of one component (dotted line) faded; the other component stained red.

galactose and mannose were identified in their respective zones (Figure 2). Fucose was found in Zone III between galactose and mannose.

The fifth zone moved more slowly than mannose, and contained hexosamine and hexuronic acid. After sodium borate was removed, the molar ratio of hexuronic acid to hexosamine in the pooled eluates of this zone was 1. Chromatography revealed two carbohydrates (Figure 3). That present in highest concentration migrated a distance identical to authentic hyalobiuronic acid,³ and stained red with *p*-dimethylaminobenzaldehyde in HCl. The other sugar, present in trace amounts, migrated a distance identical to authentic N-acetyl hyalobiuronic acid,³ and stained purple with *p*-dimethylaminobenzaldehyde in HCl.

³ Kindly supplied by Dr. Karl Meyer, College of Physicians and Surgeons.

Hydrolysates of osteoarthritic and rheumatoid synovial fluids. Glucose, galactose, fucose, mannose, hyalobiuronic acid and trace amounts of N-acetyl hyalobiuronic acid were identified in the acid hydrolysates of nondialyzable solids of these synovial fluids.

Hydrolysate of normal serum. Glucose, galactose, fucose and mannose but no hyalobiuronic acid or N-acetyl hyalobiuronic acid were found in the acid hydrolysate of the ethanol precipitate of serum.

II. Concentration of nondialyzable hexose in synovial fluid

The mean value for the nondialyzable hexose concentration in synovial fluids from 22 deceased subjects with normal joints was 0.54 mg per g (Figure 4 and Table II). Synovial fluid and

TABLE II
Levels of nondialyzable hexose in synovial fluids and sera

		Nondialyzable hexose		Synovial fluid protein mg/g
		Synovial fluid	Serum	
		mg/g	mg/g	
Normal		0.54 ± 0.11*	1.10 ± 0.09*†	25.3 ± 4.6*
Cerebral vascular accidents	54	0.61	1.90	18.3
	62	0.43	1.40	25.6
	45	0.75	1.56	29.8
	64	0.66	1.67	28.4
	70	0.69	1.68	23.5
Osteoarthritis	Mon	0.35	1.09	23.7
	Gre	0.36	1.20	23.2
	Cuo	0.45	1.15	24.6
	McN	0.53	1.18	26.7
	Tio	0.51	1.04	28.4
	Rag	0.47	1.01	30.4
	Pap	0.56	0.93	26.8
Rheumatoid arthritis	Tim	0.78		
	Cou	0.74		53.7
	Ack	1.06	1.88	62.5
	Mur Lt.	1.06	1.47	50.5
	Rt.	1.25		
	DeG Rt.	0.88	1.47	50.5
	Lt.	0.82		40.7
	Koc	0.91	1.48	
	Cas Rt.	0.78		34.8
	Lt.‡	0.44		
	Mac	0.97		53.5
	Boy Rt.	1.10		63.0
	Lt.‡	0.64		
	Swe	1.07	1.48	46.4
	Nie	1.09	1.75	43.0
Scu	0.70	1.24	52.5	
Cam Rt.	0.86	1.58	44.0	
Lt.	0.88		44.7	

* Standard deviation.

† Analysis of serum from 10 living normal subjects.

‡ Uninvolved knee.

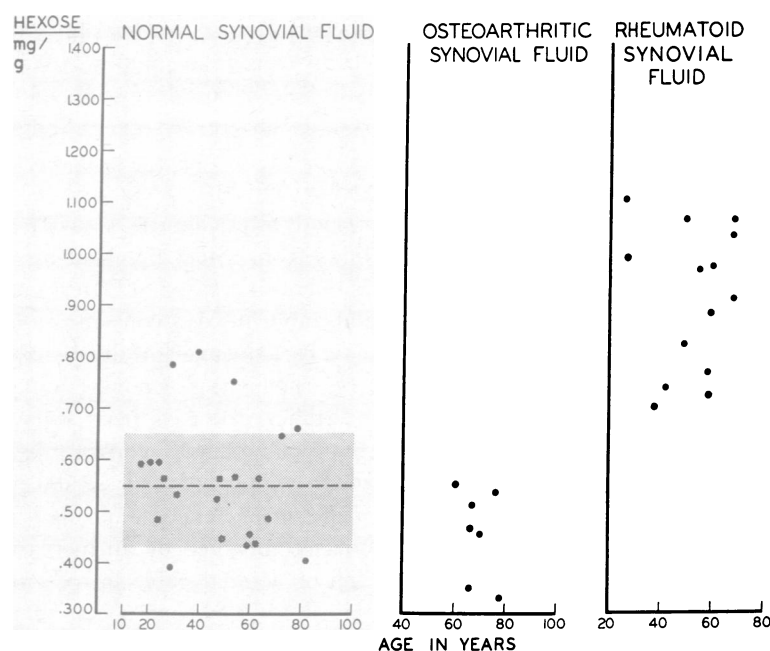


FIG. 4. NONDIALYZABLE HEXOSE CONCENTRATION OF HUMAN SYNOVIAL FLUIDS. The shaded area represents one standard deviation from the mean value (0.54 mg per g) of normal synovial fluid.

serum were also obtained from living, comatose subjects with normal knee joints but with elevated acute phase reactants due to an acute cerebral vascular accident. Although the nondialyzable hexose concentrations in these sera were elevated, hexose concentrations in the dialyzed synovial fluids were normal in four of the five cases.

The nondialyzable hexose concentration was not elevated in seven osteoarthritic fluids (Figure 4 and Table II). The volume of each of these fluids was less than 3 ml except for Tio (50 ml) and Cuo (12 ml). The nondialyzable hexose concentration in the sera of the patients with primary osteoarthritis was normal. The synovial fluid protein concentration was normal in all the osteoarthritic fluids.

The nondialyzable hexose concentration was above 0.70 mg per g in all the rheumatoid fluids from involved knees (Figure 4 and Table II). The nondialyzable hexose concentration in the sera was usually increased. The protein concentration was increased in all the rheumatoid fluids. In two patients with rheumatoid arthritis, synovial fluid was simultaneously obtained from an apparently uninvolved knee [normal physical findings, negative X-ray, volume of synovial fluid less

than 2 ml, and normal hyaluronate hexosamine concentration (17)] and from an involved knee. Nondialyzable hexose concentration was normal in the normal knee, and elevated in the involved knee (Table II).

Precipitation of protein-bound hexose by alcohol. Addition of alcohol to synovial fluids or serum precipitated the total protein-bound hexose. Values obtained by this method were similar to

TABLE III
Hexose levels of synovial fluid and serum: comparison of values obtained after dialysis or alcohol precipitation

	Hexose	
	Nondialyzable	Alcohol ppt.
	mg/g	mg/g
Synovial fluid:		
Normal	0.79 0.43 0.60 0.46	0.77 0.39 0.56 0.45
Rheumatoid arthritis	1.12	1.19
Serum:		
Normal	1.20	1.19
Rheumatoid arthritis	1.60 1.48	1.55 1.50

hexose levels obtained after dialysis of synovial fluid or serum (Table III).

Contribution of hyaluronidase-digested hyaluronate to the anthrone reaction. A solution of protein-free hyaluronate fragments containing 400 μg hexuronic acid per ml (1,000 μg hyaluronate per ml) gave an anthrone reaction equivalent to 25 μg per ml of hexose.

DISCUSSION

The carbohydrates in an acid hydrolysate of the nondialyzable solids of synovial fluid were separated by zone electrophoresis on blocks of polyvinyl chloride in borate buffer at pH 8.5. Neutral monosaccharides in the hydrolysates formed negatively charged complexes with borate and migrated in the electric field (18). Good separation of glucose, galactose and mannose was accomplished.⁴ Fucose was not completely separated by electrophoresis but was identified by paper chromatography. The use of polyvinyl chloride instead of starch as the supporting medium permitted elution without contamination with carbohydrate.

Galactose, mannose and small amounts of glucose and fucose were identified in the acid hydrolysates of dialyzed, lyophilized synovial fluids and in the ethanol precipitate of serum. The finding of glucose in these hydrolysates is of interest since in a recent discussion glucose was thought not to be one of the hexoses bound to human serum proteins (19). Hyaluronate fragments isolated from the hydrolysates of nondialyzable solids of normal and rheumatoid synovial fluids were identified as hyalobiuronic acid.

The nondialyzable hexose concentration in human synovial fluids was determined with the anthrone reagent using a solution containing equal amounts of galactose and mannose as a standard. The small volumes of viscous fluid taken for analysis were weighed rather than pipetted. The anthrone reaction of isolated protein-free hyaluronate was small (2.5 per cent of the hyaluronate concentration) and, therefore, no correction was made in the hexose levels for the hyaluronate of synovial fluid.

The mean value for the nondialyzable hexose

⁴ Free hexosamine in the acid hydrolysates was swept off the cathodal end of the block and was not recovered in these studies.

concentration in synovial fluid obtained from subjects with normal joints was 0.54 mg per g. Nondialyzable hexose levels in synovial fluids obtained from osteoarthritic knee joints were normal or decreased. Similar results were found by Decker, McKenzie, McGuckin and Slocumb (2). The nondialyzable hexose concentrations in synovial fluids from joints involved by rheumatoid arthritis were increased. Determination of the nondialyzable hexose concentration in synovial fluid may be a means for differentiating osteoarthritic from rheumatoid synovial fluid.

Studies reported here support the view (20) that the normal synovial membrane selectively adds components of serum to synovial fluid. High serum levels of protein-bound hexose were not accompanied by an increase in the concentration of nondialyzable hexose in synovial fluid obtained from normal joints of patients with acute cerebrovascular accidents and from the uninvolved knees of patients with active rheumatoid arthritis.

There is evidence that nondialyzable hexoses are bound to proteins in synovial fluid (21). Since the protein concentration was elevated in all the rheumatoid fluids, increased hexose levels were expected. However, hexose levels were not always proportional to the increased protein concentration, and it is likely that the very high hexose level in some fluids was due chiefly to an increase in proteins rich in hexose that entered the inflamed joint.

SUMMARY

1. Pooled normal human synovial fluids, and synovial fluids from joints involved by primary osteoarthritis or rheumatoid arthritis were dialyzed and lyophilized. These lyophilized solids were hydrolyzed in acid, and the carbohydrate components were separated by a combination of zone electrophoresis in borate buffer at pH 8.5, and ascending paper chromatography. Glucose, galactose, fucose, mannose, hyalobiuronic acid and traces of N-acetyl hyalobiuronic acid were found in all the hydrolysates.

2. Nondialyzable hexose concentration in normal synovial fluid and fluid from joints involved by primary osteoarthritis or rheumatoid arthritis was determined with the anthrone reagent. A hexose standard containing equal amounts of galactose and mannose was used.

3. The mean value for the nondialyzable hexose concentration in normal synovial fluid was 0.54 mg per g. Osteoarthritic synovial fluids had normal or lower levels, while rheumatoid synovial fluids had values above 0.7 mg per g. Determination of the concentration of nondialyzable hexose may be useful in differentiating osteoarthritic and rheumatoid synovial fluids.

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