CHAPTER 3

EXPERIMENTAL TECHNIQUE

3.1 TEST APPARATUS

The test apparatus for cartilage-on-stainless steel tests is based on a design by Burkhardt [31] and modified by Schroeder [23]. The device, shown in simplified form in Figure 3.1, consists primarily of a mobile table with a programmable motion controller, a vertical shaft, and a pneumatic pressure cylinder.

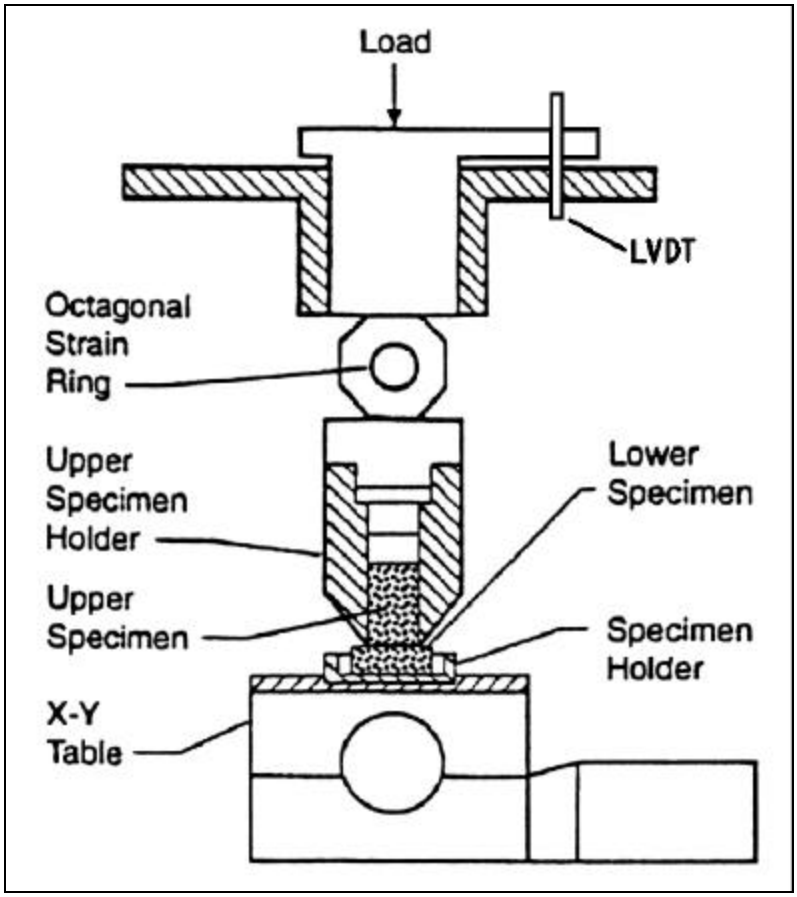

Figure 3.1: Test Apparatus

Table 3.1 shows the capabilities and flexibilities of the test apparatus.

Table 3.1: Apparatus specifications [23]

Contact System:	Cartilage on stainless steel Cartilage on cartilage
Contact Geometry:	Flat-on-flat Convex-on-flat Irregular-on-irregular
Cartilage Type:	Articular, any source
Specimen Size:	Upper: 4.0 - 6.35 mm dia. Lower: 15.0 - 25.4 mm dia.
Applied Load:	10 - 300 N
Nominal Contact Pressure:	0.6 – 2.4 MPa
Range of Motions:	Linear, oscillating Circular, constant velocity More complex patterns of motion
Sliding Velocity:	0 - 20 mm/second
Measurements:	Normal load, friction force, vertical displacement

The test parameters for this study are shown in Table 3.2.

Table 3.2: Test Parameters

Contact System:	Bovine cartilage on polished stainless steel
Contact Geometry:	Flat-on-flat
Cartilage Specimen Diameter:	6.35 mm
Applied Load: (High) (Low)	65 N 20 N
Average Pressure: (High) (Low)	2.1 MPa 0.63 MPa
Traverse:	6.5 mm
Sliding Velocity:	8 mm/second
Sliding Frequency:	40 cycles/minute
Fluid Temperature:	Ambient (20 - 25°C)
Test Duration:	3 hours
Total Cycles:	7200

For each test, a polished stainless steel disk with an R_A roughness of 100 angstroms was placed in the lower specimen holder on the X-Y table. A 6.35-mm diameter cartilage plug (bored from a bovine joint as described in Section 3.2) was secured in the upper specimen holder, and a vertical load was applied using a nitrogen tank and the pneumatic load cylinder. An IBM 8086 computer was used in conjunction with a NEAT-310 programmable motion controller to move the X-Y table back and forth along one axis. Load and friction data were obtained from a strain ring on the shaft; the signals from these gages were transmitted to two Astro-Med Dash II chart recorders (for continuous data acquisition) and an IBM 286 computer running data acquisition software (for short-term, higher frequency data acquisition). Vertical deflection of the upper cartilage sample was measured using an LVDT (linear variable differential transformer), which also supplied signals to the chart recorders and data acquisition computer.

3.2 SPECIMEN PREPARATION

The cartilage specimens for this study were obtained from 24 steers, all approximately 3 years old, with the help of Dr. Hugo Veit and the Virginia Maryland College of Veterinary Medicine. The shoulder and stifle joints from each steer were removed, labeled, and stored in a freezer at -25°C for later testing. Synovial fluid samples were taken from the same animals, and labeled for use with cartilage from the same source animal; although fluid volumes varied, each animal provided between 2 mL and 6 mL of lubricant.

Each animal was identified by a color and a number corresponding to test variables in a separate study undertaken by the College of Veterinary Medicine. The alphanumeric designation for each cartilage specimen represents the animal's color designation, its number, and the specific joint from which the specimen was taken. For example, the specimen *P3-RK* was taken from the right stifle (knee) of the steer labeled Pink-3. Because of the limited synovial fluid volumes, the fluid samples were combined for each animal into left and right portions (i.e., the left shoulder and left stifle fluid samples were combined, but kept separate from the right shoulder and right stifle samples).

Cartilage plugs for this study were cut from the stifle joints prior to testing, then re-frozen until needed. Before cutting the specimens, each joint was thawed in a bag in lukewarm water for 1-2 hours, to ensure that the bone could be easily cut. The joint was immobilized in a large vise, with the desired cartilage surface facing upward. The cartilage surface was pre-scored with two cork borers (with diameters of 6.35 mm and 9.53 mm) to prevent scoring or marring of the surface by the cutting tool. The joint was then placed on a drill press, and a 6.35 mm cutting tool was used to bore about 19 mm into the articular surface of the joint. Lubrication was provided by frequent spray from a bottle of deionized water. This cutting process was performed slowly to avoid breaking the cartilage plug inside the cutting tool.

After the plug had been bored, it was broken at the maximum depth of the bore and placed in a vial with a small amount (approximately 0.5 mL) of deionized water, and frozen for later use at -25°C. Four to six cartilage plugs were cut from each stifle joint. After all plugs were cut from the joint, the joint was then replaced in the freezer and stored at -25°C.

The stainless steel lower specimens were cut from a 303 stainless steel rod, 25.4 mm in diameter. Each disk was 6.35 mm thick, and polished to an approximate surface roughness of R_A = 100 angstroms. Each disk was rinsed with deionized water and air-dried thoroughly to remove particles after polishing.

3.3 LUBRICANT PREPARATION

The three lubricants used in this study were buffered saline solution, hyaluronic acid in saline, and synovial fluid. The phosphate-buffered saline solution, chosen as a reference lubricant, was supplied by Dr. E. M. Gregory in the Department of Biochemistry. To produce the hyaluronic acid solution, 0.375 weight percent human umbilical cord hyaluronan, obtained from the Sigma Chemical Company, was dissolved in the reference buffered saline solution. In each synovial fluid test, the fluid was chosen from the same steer from which the tested joint was obtained. The hyaluronic acid solution and synovial fluid were both stored at -25°C.

3.4 PROCEDURE FOR *IN VITRO* EXPERIMENTS

The test apparatus is described in detail in Section 3.1. Figure 3.2 illustrates the overall process followed for each test. Before each wear test began, the cartilage plug and lubricant were fully thawed for at least 30 minutes. A stainless steel disk was anchored securely in the lower specimen holder, and 1 mL of the lubricant added to the surface of the disk. A rubber gasket prevented the lubricant from leaking from the surface. The cartilage plug was then placed in the upper specimen holder, which was fastened onto the shaft of the test device.

After the cartilage specimen was fastened in place, the shaft was lowered until the cartilage surface came into contact with the stainless steel disk. An LVDT displacement reading was obtained on the chart recorder to provide an initial vertical displacement measurement. The computer-controlled X-Y table was set in oscillating motion, and the load (65 N or 20 N) was applied. The first computer data file was collected within three cycles of the start of motion; each of these computer output files contained three seconds of normal force, tangential force, and vertical displacement data taken at a sampling frequency of 250 Hz. During the test, more computer output files were generated after 30, 60, 120, and 180 minutes.

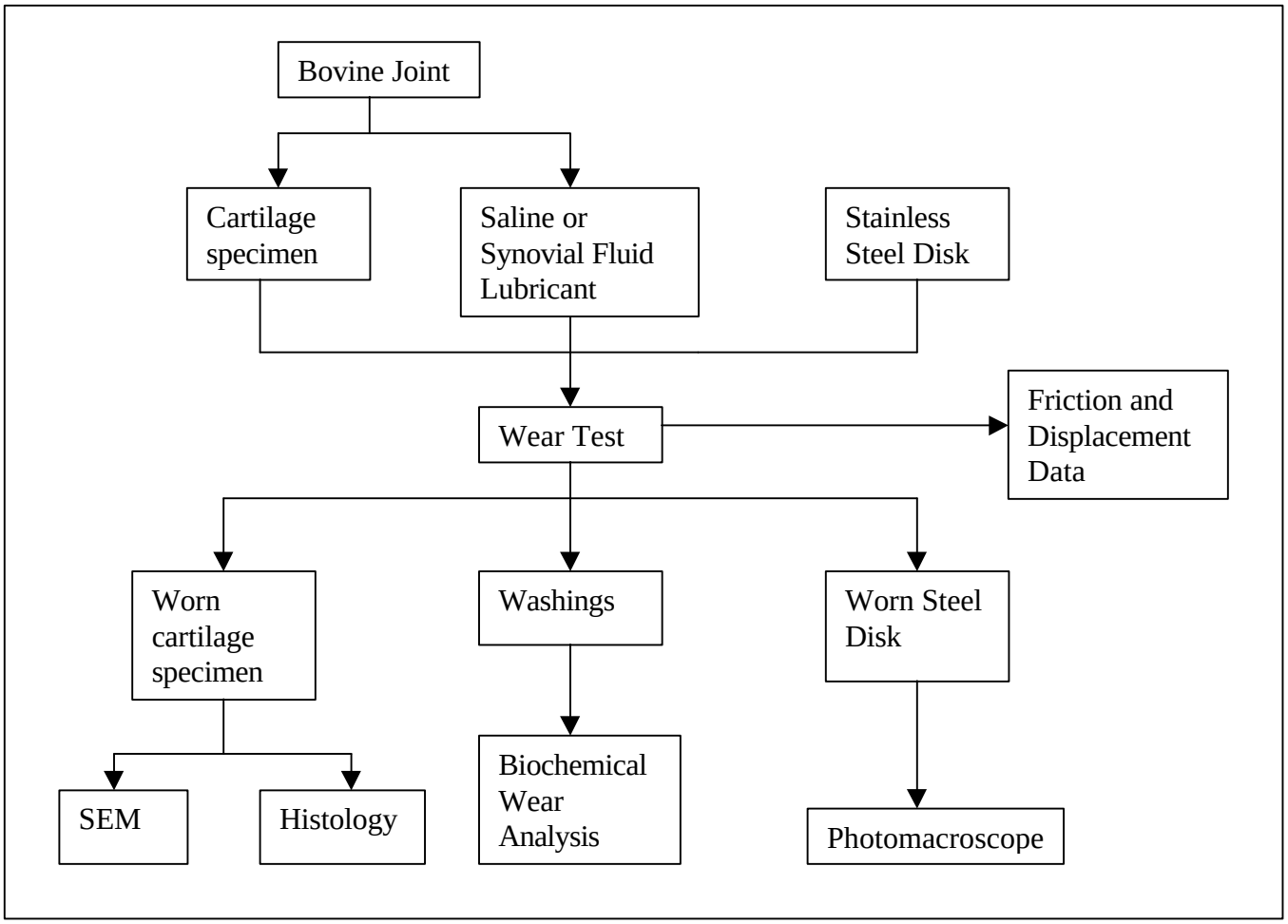

Figure 3.2: Test Procedure Diagram

At the end of each test, the sliding motion was interrupted while the specimen was still under load, so that a final vertical displacement measurement could be obtained. One minute after the X-Y table was halted, the load was removed and the shaft raised. The disk and cartilage surfaces were rinsed with 3 mL deionized water, and the collected washings were stored at -25°C until needed for analysis.

The cartilage plug from each test was saved for either scanning electron microcopy (SEM) or histological sectioning and staining. Those specimens that were reserved for SEM were truncated with a razor blade approximately 4 mm from the cartilage surface, parallel to the surface, and stored in fixative solution at 5°C. The fixative consisted of 5% glutaldehyde, 3% formaldehyde, and 2.75% picric acid in 0.1M sodium cacodylate buffer at a pH of 7.3 to 7.4. Histology specimens were sliced perpendicularly to both the cartilage surface and the direction of sliding, and each half was placed in formalin solution for later sectioning. Table 3.3 shows the specimens, loads, and lubricants used in each test.

Table 3.3: Overview of Tests

Test	Cartilage Source	Load	Lubricant
1	O1-RK	65 N (high)	Saline Solution
2	O1-RK	65 N	Synovial Fluid
3	O1-RK	20 N (low)	Saline Solution
4	O1-RK	20 N	Synovial Fluid
5	O1-LK	65 N	Saline Solution
6	O1-LK	65 N	Saline + Hyaluronic Acid
7	O1-LK	20 N	Saline Solution
8	O1-LK	20 N	Saline + Hyaluronic Acid
9	G3-RK	65 N	Saline + Hyaluronic Acid
10	G3-RK	65 N	Synovial Fluid
11	G3-RK	20 N	Saline + Hyaluronic Acid
12	G3-RK	20 N	Synovial Fluid
13	W11-RK	65 N	Saline Solution
14	W11-RK	65 N	Synovial Fluid
15	W11-RK	20 N	Saline Solution
16	W11-RK	20 N	Synovial Fluid
17	P7-LK	65 N	Saline Solution
18	P7-LK	65 N	Saline + Hyaluronic Acid
19	P7-LK	20 N	Saline Solution
20	P7-LK	20 N	Saline + Hyaluronic Acid
21	G9-LK	65 N	Saline + Hyaluronic Acid
22	G9-LK	65 N	Synovial Fluid
23	G9-LK	20 N	Saline + Hyaluronic Acid
24	G9-LK	20 N	Synovial Fluid

3.5 WEAR ANALYSIS PROCEDURE

The amount of wear in each test was determined by measuring the amount of hydroxyproline in each test washing. This procedure was performed in the lab of Dr. E. M. Gregory in the Department of Biochemistry at Virginia Tech. The protocol for this technique is presented in Appendix D.

After each test, the lubricant was removed from the surface of the stainless steel disk with a plastic transfer pipette. The surface was then rinsed with 3 mL of deionized water, and stored in a small vial at -25°C. These specimens were accumulated and tested for cartilage wear in two batches of 12 specimens each.

On the day of the wear analysis, the vials containing the washings were transported frozen to the biochemistry department, and then thawed. A 1 mL sample from each of the synovial fluid specimens was also allocated for wear analysis, so that any hydroxyproline present in the synovial fluid before testing would not provide a misleading wear measurement.

Each fluid sample was first shell-frozen in a thick-walled culture tube, and lyophilized overnight. What remained of each specimen afterwards was a white or yellow solid, which was rinsed into a glass hydrolysis vial with 1.5 mL of 6M hydrochloric acid. Each specimen was then sealed inside its vial, and hydrolyzed for 24 hours at a temperature of 105°C.

Each specimen was centrifuged after hydrolysis, and the supernatant fluid was decanted and diluted to a total volume of 2 mL with 6M hydrochloric acid. Two or three samples were then taken from the hydrolysate, in volumes of 100 μL, 50 μL, and, in some cases, 25 μL. Each sample was neutralized with an equal volume of 6M sodium hydroxide, and diluted with distilled water to a total volume of 200 μL.

0.4 mL of isopropanol was added to each sample, followed by 0.2 mL of the chloramine-T reagent (reagent A, described in Appendix D). The solution was incubated at room temperature for 5 minutes, and 2.5 mL of the DAB reagent (reagent B, described in the appendix) was added. The mixture of these reagents with hydroxyproline resulted in a color that was measured with a spectrophotometer. The absorbance at a wavelength of 558 nm was used to determine the concentration of hydroxyproline present in the sample.

A hydroxyproline standard curve was produced using a set of samples containing 0 μg to 1 μg of hydroxyproline. This set of known standards was taken through the same procedure to generate a calibration curve in terms of absorbance vs. hydroxyproline mass. The two hydroxyproline standard curves are shown in Figures 4.13 and 4.14. After obtaining each absorbance and hydroxyproline measurement, the result was multiplied by a value corresponding to each sample's dilution. It was assumed for the purposes of

calculation that hydroxyproline accounts for 2% of the total weight of hydrated cartilage [21, 22].