METHODS FOR MEASURING THE ULTRASONIC ABSORPTION OF AQUEOUS SOLUTIONS OF DEOXYRIBONUCLEIC ACID

BY

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THESIS

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY CHRISTOPHER LAWRENCE CHRISTMAN

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Chapter 1

INTRODUCTION

The mechanism by which energy is absorbed from acoustic waves passing through biological tissue is not well understood (Kessler [1968]). Kessler [1968] has suggested that molecular interactions may be responsible. Several different conformal structures exist for biological macromolecules in solution. By comparing the ultrasonic absorption of molecules of similar structure in solution, it may be possible to identify similarities which may ultimately lead to a better understanding of the structure and function of biological macromolecules as well as to a better understanding of the ultrasonic absorption process. Kessler [1968] has summarized these possible configurations. He measured the absorption properties of Bovine Serum Albumin and compared them to Polyethylene Glycol (PEG). For the present investigation, which is a continuation of Kessler's work, ultrasonic absorption and velocities measurements were made in aqueous solutions of Deoxyribonucleic Acid (DNA), an important biological macromolecule having a structure much different from proteins.

In Chapter 2, several mechanisms responsible for the ultrasonic absorption in liquids will be discussed. The absorption properties are considered to be intimately related to the structure of the molecules making up the solution and to their internal energies. Therefore, by changing the structure of the DNA, it is possible to gain some insight into the mechanism for its observed absorption characteristics. Chapter 3 describes how this can be done by changing the pH of the DNA solution.

Since high molecular weight DNA is expensive and large quantities; i.e., one liter, are needed for measurements with the presently available instrumentation, it is desirable to develop a measuring system which can

utilize small quantities and obtain the most information in the shortest period of time. Suitable instrumentation was designed and developed to measure simultaneously the absorption coefficient at several different frequencies. Comparison measurements were made with this new instrumentation on standard reference materials and the instrumentation was found to be acceptable. Solutions of two different types of DNA were used at frequencies from 5.07 MHz to 19.3 MHz in the pH range from 7.4 to 1.9. In summary, the purpose of this investigation was to measure the ultrasonic absorption coefficient of aqueous solutions of DNA and to develop ultrasonic measuring techniques for obtaining such data over a broad frequency range in a simultaneous manner rather than the conventional "one frequency at a time" method.

Chapter 2

ULTRASONIC ABSORPTION

Propagation of ultrasound through a fluid produces fluctuations in both the pressure and density of the medium. To understand the consequences of these changes, the fluid is assumed to be composed of a large number of particles, each of which is small enough so that the physical properties, such as pressure, density and sound velocity are constant over it. The particle is also considered to be large enough to ignore intermolecular processes. When a longitudinal plane wave passes through the medium, the fluid's particles are displaced and the elastic restoring force, resultion from the finite pressure, tends to return the particles to their equilibrium position. By applying Newton's second law, conservation of mass and assuming a thermodynamic equation of state, the acoustic plane wave equation is derived as (Kinsler and Frey [1962]),

$$\frac{\partial^2 \xi}{\partial t^2} = c^{*2} \frac{\partial^2 \xi}{\partial x^2} \qquad (2\text{-}1)$$

where ξ is the particle displacement and c^* is the complex wave velocity. If the medium is dissipative, energy will be removed from the wave as it propagates. The absorption coefficient is a measure of the amount of energy lost. Assuming a lossy medium, the solution of equation (2-1) has the following form:

$$\xi = \xi_o e^{-\alpha x} e^{-j(\omega t - \beta x)} \qquad (2\text{-}2)$$

where α is the absorption coefficient, ω is the angular frequency and β is the propagation constant of the medium. The acoustic pressure, density and particle velocity are related to the first derivative of the particle displacement (Kinsler and Frey [1962]). These properties will therefore have

the same exponential relationships as equation (2-2) but will differ by a constant.

One major loss mechanism responsible for absorption in liquids is viscosity. By using Miexner's general stress-strain relation (Mason [1965]), it is possible to derive an expression for the acoustic velocity of the medium. An equivalent expression can be derived by substituting equation (2-2) into the plane wave equation. The result, which is a function of α, shows that the velocity must be a complex number if α is finite. By equating the real and imaginary parts for both expressions of the velocity, the absorption for low frequencies is given by

$$\frac{\alpha}{f^2} = \frac{2\pi^2}{\rho_o c^3} \left[\eta_v + \frac{4}{3}\eta_s\right] \qquad (2\text{-}3)$$

where f is the frequency of propagation, ρ_o is the equilibrium density of the medium, c is the velocity, η_s is the shear viscosity, and η_v is the volume viscosity (Mason [1965]). Stokes in 1845 recognized the existence of a volume viscosity but since he had no way of measuring it, he assumed it was zero. The only way to determine η_v for a particular medium is to measure its absorption.

Thermal losses can also play an important role in explaining a fluid's absorption. Since the propagation of an ultrasonic wave is an adiabatic process, a temperature gradient will exist between regions of compression and rarefaction in the wave. When heat flows from areas of higher temperature to areas of lower temperature, energy will be lost resulting in the absorption given by

$$\frac{\alpha}{f^2} = \frac{2\pi^2}{\rho_o c^3} (\gamma-1) \frac{K}{c_p} \qquad (2\text{-}4)$$

where K is the thermal conductivity, c_p is the specific heat at constant pressure, and γ is the ratio of c_p to the specific heat at constant volume.

The classical absorption is defined as the sum of the absorption due to shear viscosity and to thermal conductivity. The excess absorption which is the difference between the measured absorption and the classical absorption is non-zero for most liquids, indicating the existence of a volume viscosity and other absorption processes. Frequency dependent mechanisms, called relaxation processes, have been invoked to explain the existence of a volume viscosity.

A structural relaxation process occurs when the sinusoidal pressure variations of an acoustic wave produce volume changes in the medium. Eyring [1936] proposed a theory to explain shear viscosity by postulating the existence of holes in liquids. Later he was also able to explain bulk viscosity by a similar argument. If the volume decreases discontinuously from one constant value to another, the equilibrium between the number of holes and the number of molecules changes. This process will not occur instantaneously but will exhibit an exponential relation. The time constant associated with this process will equal the time needed for the pressure to reach 1/e of its final value. The bulk viscosity, which is the volume viscosity associated with a structural relaxation process, is the product of the relaxation modulus of the material and the relaxation time constant.

Since the same molecular bonds must be broken to fill the holes associated with both shear and bulk viscosity, it seems reasonable to assume they will have approximately the same value at a given temperature. Similarly, they should follow the same temperature dependence. Assuming the activation energy and entropy are equal for both shear and bulk viscosity, Hirai and Eying [1958] have shown that a standard value for the observed absorption

divided by the classical absorption for liquids is 5.1. Therefore, structural relaxation processes are characterized by liquids whose observed absorption is between one and five times the classical absorption and by liquids in which this ratio does not change as a function of temperature.

An example of a material which exhibits a structural relaxation process is water. The observed absorption in water is approximately three times the classical value. Thermal effects play no significant role in the acoustic absorption of water since water becomes isothermal at 4^{o} C indicating that no thermal gradient will exist as the wave propagates through the medium. Hall [1948] proposed the first structural model for the ultrasonic absorption in water. He assumed water consisted of two states. State one was characterized by larger volume and lower energy than state two and represented a tetrahedral ice-like arrangement. State two was characterized by a smaller volume and higher energy than the first state and corresponded to water's close packed arrangement. He assumed an equilibrium between the transitions from one state to another. Hall was able to calculate the difference in volume between the states to be 47%. His model predicts a relaxation time of 1.6×10^{-12} sec. at 30^{o} C which is well above the present range of measurements.

Thermal relaxation processes occur when the temperature gradient produced by the acoustic wave perturbs the rotational and vibrational degrees of freedom of a molecule. During a condensation when the temperature rises, a certain percentage of the molecules can change their rotational or vibrational energies by extracting energy from the wave. There will be a time lag between the sinusoidal temperature variations and the corresponding changes in degrees of freedom. This time lag results in an energy loss and thus in an excess absorption for the medium. Thermal relaxation processes are

characterized with ratios of the observed absorption to the classical absorption which are twenty or greater and which have no correlation with temperature. These processes occur most frequently in non-associated non-polar liquids such as benzene.

Chapter 3

PHYSICAL PROPERTIES OF DEOXYRIBONUCLEIC ACID

Deoxyribonucleic acid, DNA, is a particularly important macromolecule belonging to a larger class called nucleic acids. The building block of the nucleic acids is the polyester chain to which various groups may be attached to produce different molecules. In the specific case of DNA, phosphoric acid serves as the acid portion of the ester while a sugar, D-2 deoxyribose, is its alcohol component (Morrison and Boyd [1966]). The D-2 deoxy prefix indicates that the OH group is missing from the second carbon position of the sugar. The structure is completed when base units are attached to this polyester chain. A base-sugar unit is called a nucleoside while a nucleotide consists of the base, sugar and phosphoric acid groups.

Four different bases are found in DNA. Two of the bases, adenine and guanine, contain the purine ring structure; and two, thymine and cytosine, have the pyrimidine ring structure. Watson and Crick [1953] proposed their famous double helix model to explain the structural configurations of DNA. It was envisioned to consist of two right hand hexical chains with the bases occupying the interior of the helix. Spatial considerations would allow only a purine ring base to pair with a pyrimidine ring base. Specifically, adenine was hydrogen bonded to cytosine and guanine was hydrogen bonded to thymine, but all other conceivable pairs of the four bases were excluded. This model was successful in explaining how the DNA molecule can store and transmit the genetic code. Each strand of the double helix is the mirror image of the other and both strands contain exactly the same information. One strand can thus be used to direct the manufacture of proteins while the other can serve as a template for the construction of a new double helix.

The process which results in the separation of one polynucleotide chain from its mirror image is called denaturation. Single-stranded DNA resembles randomly coiled highly flexible chains which have considerable rotational freedom. In contrast, undernatured or native DNA is rigid, highly extended and will allow only gentle coiling along its length. One way of denaturing the molecule is by changing the pH of a solution of the DNA. In a specific acidic range, the ademine-thymine group becomes protonated when a hydrogen ion is added to these bases. This reaction cleaves the normal hydrogen bonds between ademine and thymine. As the pH is reduced, the guanine group is protonated and undergoes a tautomeric shift which changes its structure. Protonated guanine is electrostatically charged with the proper polarity to repulse its neighboring cytosine base. As a result, the DNA macromolecule denatures. Michelson [1963] reported that an acid titration of a DNA solution will irreversibly change the molecule's structure when 75% of its hydrogen bonds are broken. These changes will typically occur for pH values below 2.6.

In the alkaline region, removal of a proton from the guamine-thymine units will only remove one of its two hydrogen bonds. The molecule will eventually denature due to the increased electrostatic repulsion from the phosphate groups as the pH increases. Ionic solutions are used to inhibit this process by shielding the electrostatic charges of the phosphate groups. The specific ionic solution used in this study was SSC which consisted of 0.15 moles of sodium chloride and 0.015 moles of sodium citrate. Even when SSC is used as the solvent, DNA solutions will be completely denatured at pH values greater than 12.

The denaturation process of DNA in solution can be studied by investigating its ultraviolet absorption spectra. Polynucletides character-

istically exhibit the hyperchromatic effect. This effect occurs when the total ultraviolet absorption for a material is less than the sum of the absorption which could be attributed to each one of its individual parts. Michelson [1963] has explained hyperchromism for DNA in the following manner. Its bases are stacked in layers which are close enough for interactions by π electrons. These π bonds have their own distinctive ultraviolet absorption spectra which is not simply the summation of the absorption due to its component parts. As the hydrogen bonds between base residues are broken, these π bonds are disrupted and the ultraviolet absorption increases. The hyperchomatic effect can be used to penpoint where the DNA of a particular ionic solution denatures.

The Beckman Model DU Spectrophotometer was used for all ultraviolet measurements. Its operation is based on Beer's Law which is given by

$$OD = \varepsilon_\lambda \ell c \qquad (3\text{-}1)$$

where OD is the optical density of the solution, ε_λ is its extinction coefficient measured at a wavelength, λ, c is its concentration, and ℓ is the optical path length. All spectrophotometric measurements were made at a wavelength of 260 mu. An experiment was preformed to measure the extinction coefficient of a SSC solution of Salmon Sperm DNA as a function of pH. Two different concentrations, one at 22.6 μg/ml and the other at 21.7 μg/ml, were used for the base titration. The measured OD was subtracted from the OD of the solvent and then the extinction coefficient was calculated. The results are shown in Figure 3.1. An acid titration was also conducted for solutions containing DNA concentrations of 23.6 μg/ml and 19.9 μg/ml as shown in Figure 3.2. Since the ultraviolet absorption is directly related to the extinction coefficient, these curves show that Salmon Sperm DNA is

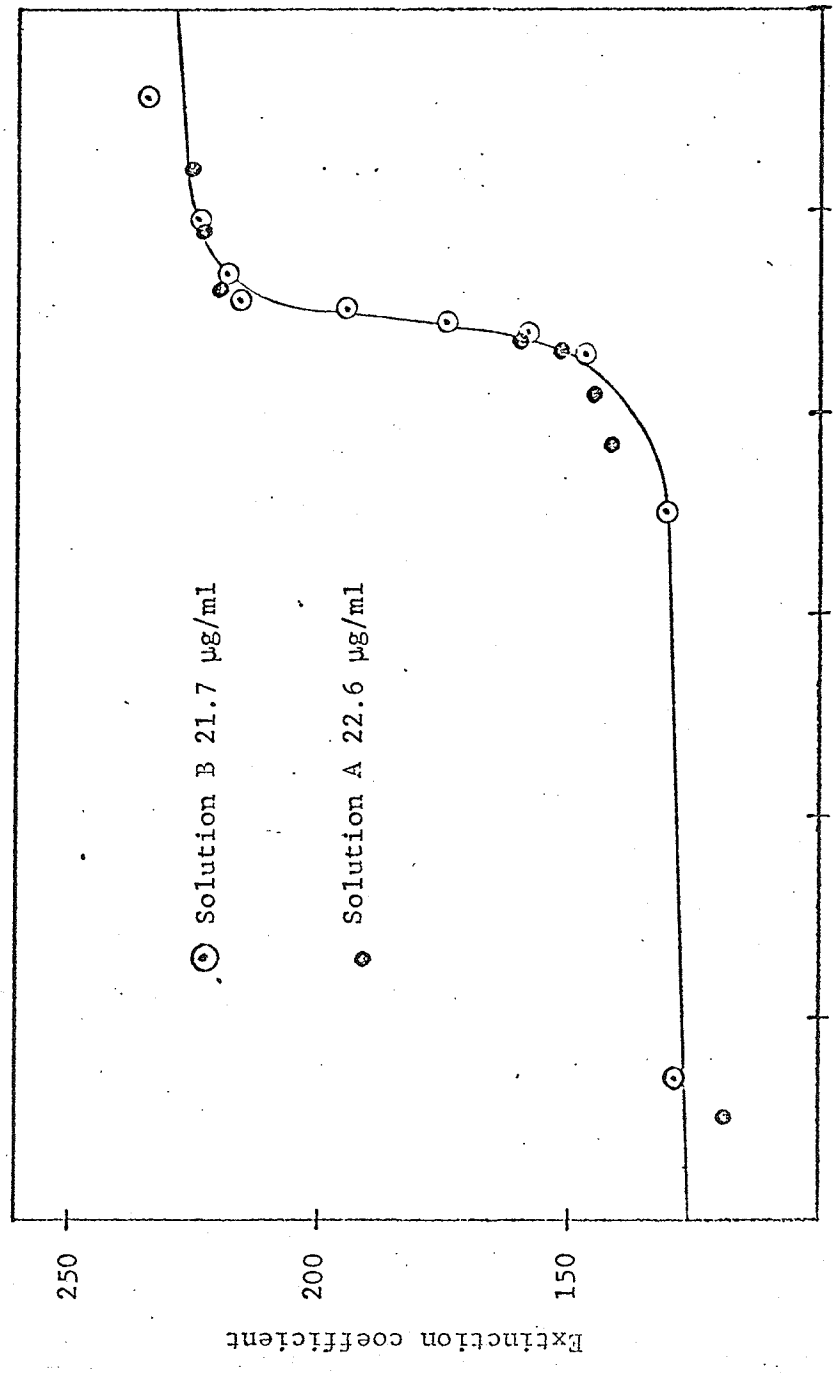

Figure 3.1. Base Titration of Salmon Sperm DNA.

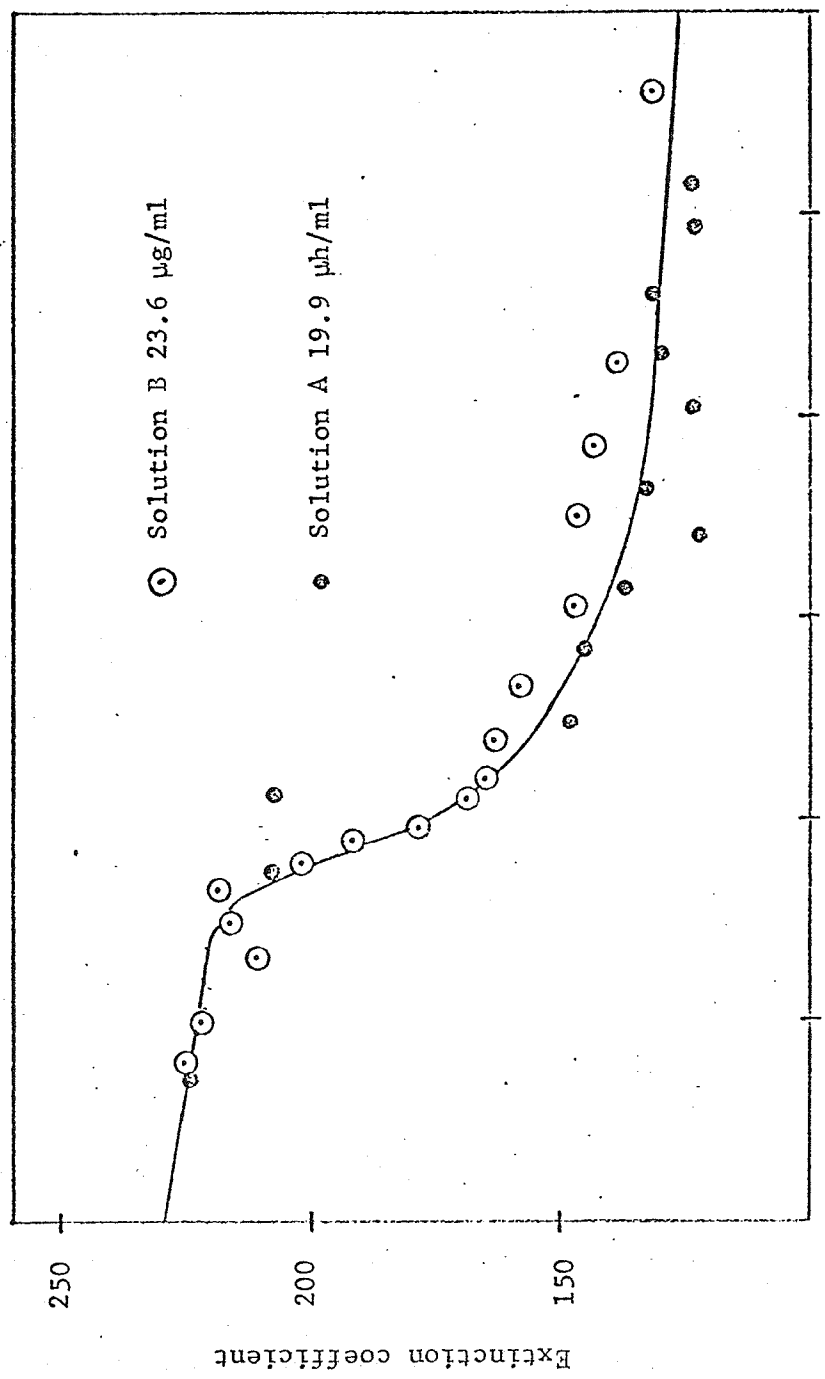

Figure 3.2. Acid Titration of Salmon Sperm DNA.

completely denatured for pH values below 2.0 and above 12.0.

Many different types of DNA are commercially available. Calf Thymus DNA, obtained from the Mann Research Laboratory, was used for one set of absorption measurements. This material was allowed to go into solution by stirring at 10^{o} C. The Calf Thymus DNA would go into solution in 24 hours. Ten drops of chloroform were added to impede the growth of bacteria. The solution was stored in the refrigerator until it was used. All concentration measurements were made using the spectrophotometer. Equation (3-1) was used to calculate the concentration after ε_{λ} was experimentally determined. The extinction coefficient was measured at pH 12 and yielded a value of 219 for the Calf Thymus DNA.

Highly polymerized Salmon Sperm DNA, which was obtained from Calbiochem, Los Angeles, was also used for acoustic absorption measurements. Its molecular weight was higher than the Calf Thymus DNA previously described. The Salmon Sperm DNA was put into a solution of SSC by stirring at 10^{o} C. Chloroform was also added to limit bacterial growth and three weeks were allowed for the DNA to go into solution. Protein complexes formed from the impurities found in the DNA and were eventually removed through the following purification process. The DNA solution was centrafuged for twenty minutes at 20,000 rpm. All the large aggregates were removed with this step. The solution was then passed through a three stage filtration process in which No. 1 Whatman filter paper was used in the first stage, a 5. μ millipore filter was used during the second stage, and a 0.9 μ millipore filter was used in the final stage. Chloroform was again added and the solution was stored at 10^{o} C until used. Josse and Eigner [1966] have reported that 130×10^{6} molecular weight DNA can be filtered through a 0.22 μ millipore filter without damage. Since the molecular weight is approximately one

million for the Salmon Sperm DNA, this purification process did not effect the structure of the native DNA. The concentration measurements made were similar to the technique used for the Calf Thymus DNA. The extinction coefficient for the Salmon Spern DNA was given by the manufacturer to be 224 for solutions of pH 12.

Chapter 4

INSTRUMENTATION

Two different systems were used for measuring the absorption coefficient and the sound velocity; one operation from .56 MHz to 12 MHz and the other from 1.1 MHz to 163 MHz. The low frequency system is based on a comparison technique developed by Carstensen [1954]. The measuring tank, shown in Figure 4.1, consists of two chambers separated by an acoustic window. The material used for the window is chosen so its acoustic impedance approximately matched that of the sample in order to minimize reflections. Polythelyne sheet 0.003 in. thick stretched over the frame, treated with hot tap water and then restretched to insure a flat surface provides an acceptable window. One chamber contains the reference liquid while the other chamber holds the sample liquid. Distilled water was used in the present study as the reference because its acoustic properties are well known and because its speed of sound and the acoustic impedance closely match the fluids investigated. The transducers are attacked to a movable carriage which is driven through a gear train by a synchronous motor. The position of the carriage can be determined to 10^{-5} m by a Gaertner Model M303 micrometer slide equipped with a traveling microscope.

The transducers used were a matched pair of two inch diameter lead zirconate titanate (PZT) ceramic discs. These polycrystalline ceramics are made piezoelectric by a polarizing process (Berlincourt [1964]). The thickness resonance frequency of such piezoelectric discs is given by

$$f_n = \frac{(2n-1)c}{2d} \quad , \; n = 1,2,\ldots \qquad (4\text{-}1)$$

where f_1 is the fundamental resonance frequency, f_n are the odd harmonics, c is the velocity of propagation in the transducer material and d is its

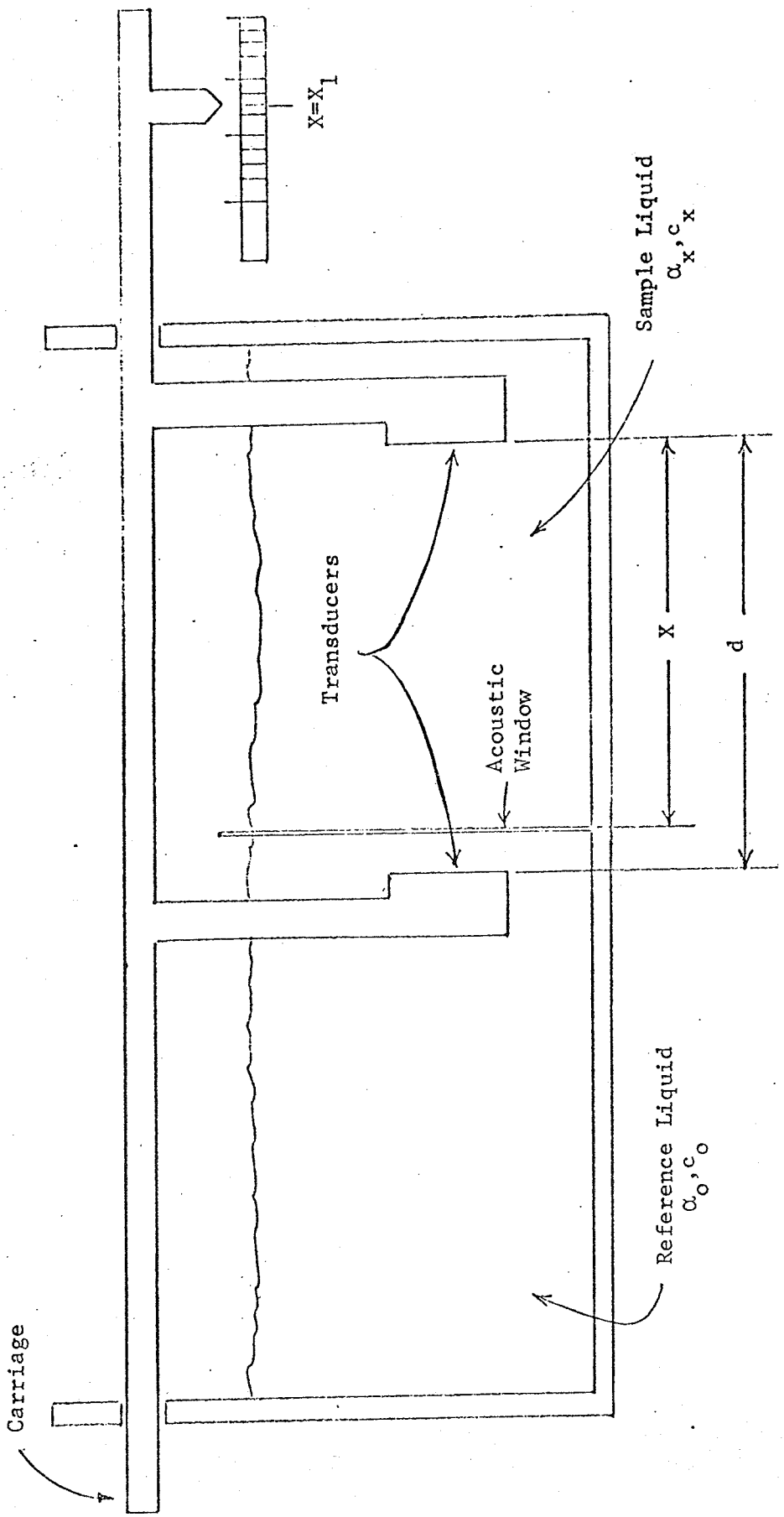

Figure 4.1. Low Frequency System (Kessler [1968]).

thickness (Kinsler and Frey [1950]). Equation (4-1) states that a resonance occurs when the thickness of the vibrator equals an odd multiple of half wavelengths. Table 4.1 shows the fundamental and some of the possible harmonics which were experimentally determined for the pair of transducers used in the low frequency system.

The alignment of the transducers is a critical factor which limits the precision of the measurement. To achieve the best possible parallelism, the transducers are aligned at the highest operating frequency. Since the beam is most directional at these frequencies, the parallelism becomes less important at lower frequencies. Each transducer is attached to the carriage by a modified military "astro compass" which allows motion along the three cylindrical coordinates. Once the transducers are aligned the "astro compass" can be locked to provide a rigid supporting structure.

The temperature of the low frequency system is maintained by a water jacket surrounding the tank. The jacket water is pumped continuously through a reservoir which is regulated by a Yellow Spring Model 71 binary controller. Kessler [1968] reported that the temperature of the chambers can be kept within $\pm$ .02° C. Circulating pumps mix both the reference and the sample liquids to reduce further any thermal gradients present.

The transducers are arranged a fixed distance apart on the carriage which traverses the length of the chambers. Thus the path length in the sample fluid changes as the carriage moves and the following equation describes the average pressure at the receiving transducer:

$$p = p_o e^{-\alpha_o (d-x) - \alpha_x x} \qquad (4\text{-}2)$$

where α_o is the absorption coefficient of water, α_x is the absorption coefficient of the sample, d is the separation distance of the transducers,

Table 4.1

Resonance Frequencies for the Low Frequency System's Transducers

Diameter	2"
Thickness	0.396 cm
c - velocity	4.46×10^5 cm/sec.
f_1 - fundamental frequency	0.56373 MHz

Harmonic	Frequency (MHz)
3	1.6912
5	2.8186
7	3.9461
9	5.0735
11	6.2010
13	7.3284
15	8.4559
17	9.5833
19	10.711
21	11.838

and x is the distance from the window to the receiving transducer. Since the transducers are piezoelectric, the output voltage developed by the receiving transducer will be directly proportional to the sound pressure on its face. The excess absorption, α_{ex}, which equals $\alpha_x - \alpha_o$, is given by the following equation:

$$\alpha_{ex} = \frac{\ell n\, E_2 - \ell n\, E_1}{x_2 - x_1} \qquad (4\text{-}3)$$

where E_2 is the receiving transducer output voltage at x_2 and E_1 is that voltage at x_1.

It is often possible to use multiple transits of the acoustic pulse between the transducers to measure the absorption. As the transmitted pulse strikes the receiving transducer some energy is transmitted into the ceramic material, but because of the impedance mismatch between water and the ceramic most of the energy is reflected. If the absorption of the sample is low the pulse will contain sufficient energy to travel back and forth between transducers several times before being significantly attenuated. The "second pulse" is defined as the pulse which has traveled the path between the transducers three times. When using the second pulse, the absorption is given by equation (4-3) divided by a scale factor, c_n, which accounts for the increased path length, e.g., c_n equals three for the second pulse. One of the chief problems with this technique is the interference produced by reflections from the window. The third and fourth pulses could be used in a similar manner but the window reflection problem is even more severe.

Velocity differences as small as 0.01% of the total velocity can accurately be measured with the low frequency system (Carstensen [1954]). Distilled water is again used as the reference since its velocity is well known. Since the wavelength distance in the reference liquid and in the

unknown liquid are different, the number of wavelengths between transducers is given by

$$n = x/\lambda_x + (d-x)/\lambda_o \qquad (4\text{-}4)$$

where λ_x is the wavelength in the sample and λ_o is the wavelength in water. If the number of wavelengths is changed by m, after the carriage has been moved Δx, Kessler has shown the velocity in the unknown liquid to be:

$$c_x = \frac{c_o}{1 \pm \frac{mc_o}{\Delta x f}} \qquad (4\text{-}5)$$

where c_o is the velocity of water and f is the frequency.

The high frequency system shown in Figure 4.2 provides another method for measuring ultrasonic absorption. The system is based on Pellam and Galt's [1946] equipment and consists of a water jacketed stainless steel cylinder containing a movable piston. The orientation of the transducer mounted on the piston is fixed so the parallelism is controlled by aligning the lower transducer. A circulating pump with suitable inlet and outlet vents insures complete mixing of the sample liquid. The temperature of the system is controlled by a Yellow Spring Model 72 "proportional" temperature controller which can maintain the temperature to within $\pm$ 0.01° C. The piston is driven by a synchronous motor which is attached to a worm gear through a gear reducer. Absorption measurements are made as the piston travels through the cylinder, where α is the absorption coefficient, given by

$$\alpha = \frac{\ell n\, E_2 - \ell n\, E_1}{x_2 - x_1} \qquad (4\text{-}6)$$

where E_2 is the output voltage when the transducers are separated by a distance x_2 and E_1 is the voltage at x_1.

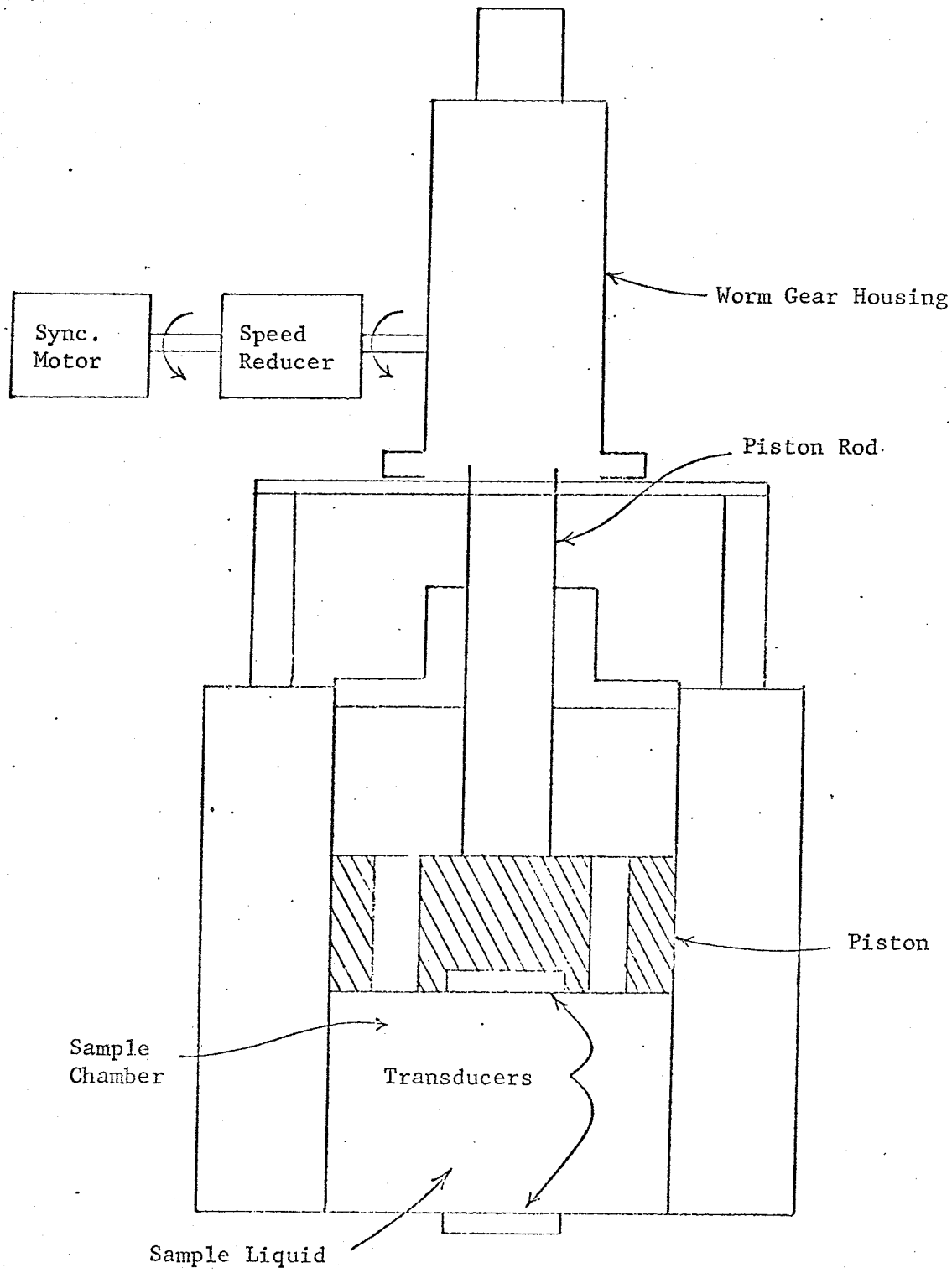

Figure 4.2. High Frequency System (Kessler [1968]).

The velocity can also be measured with the high frequency system, since the output and the input signals are shifted in phase because of the delay caused by the pulse propagation through the medium. If the piston is moving when the input and output signals are mixed, an interference signal is produced which is periodic in the time interval, Δt, during which the piston moves one wavelength. Kessler [1968] has shown that the following equation gives the velocity in the medium:

$$c = \frac{\nu_p \omega_o \Delta t}{2\pi} \qquad (4\text{-}7)$$

where ν_p is the velocity of the piston and ω_o is the angular frequency of the acoustic wave.

Kessler [1968] reports that measurements were made with the high frequency system on water to 80 MHz with an accuracy of 3.0%. Above 100 MHz the accuracy decreased to 5.5%. Diffraction effects, which will be discussed in Chapter 5, determine the lowest frequency which can be used for measurements. The upper frequency limit is determined by the orientation of the transducers. Parallelism in the low frequency system again limits the frequency range. This problem is more severe with the low frequency system because as the carriage moves, bending and twisting motions can change the transducer's orientation.

The function of the electronic instrumentation used for both measuring systems is to generate a pulsed RF signal which excites the transmitting crystal and to detect the received pulse and record its amplitude.

A block diagram of the transmitting circuitry is shown in Figure 4.3. The signal from an RF oscillator whose frequency is monitored with a Systron Donner Model 1037 frequency counter is applied directly to an Arenberg gated amplifier. Its RF output pulse is tuned by an internal capacitance and a

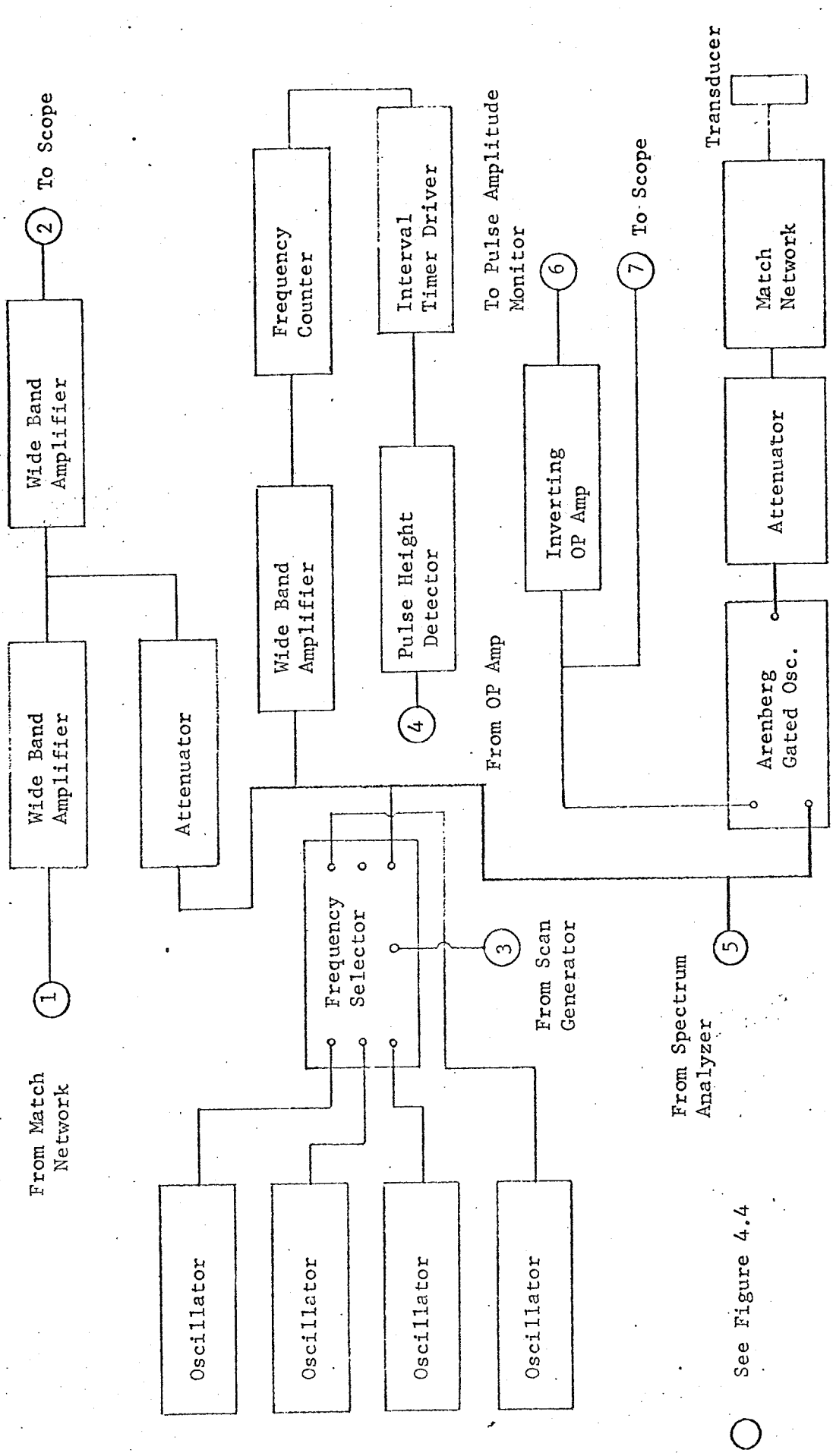

Figure 4.3. Block Diagram for the Transmitting Pulse Circuitry.

plug-in coil which operates over a specific frequency range. The range can be altered by changing coils. The circuit is sufficiently broad-banded so that one coil covers the frequency range 5.0 MHz to 12. MHz. The output pulse is connected to an attenuator which reduces standing waves produced by impedance mismatches. The pulse is then applied to a matching network before being applied to the transducer.

The block diagram for the pulse receiver is shown in Figure 4.4. An electric potential is produced when the acoustic wave strikes the receiving transducer. The resulting signal is processed through a matching network and an attenuator and then is applied directly to the Hewlett Packard Model 8552A/8553L Spectrum Analyzer. The analyzer displays the amplitude of the input signal as a function of frequency on an oscilloscope. Its center frequency control allows the horizontal midpoint of the scope to be adjusted to any frequency from 1 KHz to 110 MHz. The frequency range is controlled by the scan width per division setting and can be adjusted from plus or minus 50 MHz of the center frequency to plus or minus 1 KHz of the center frequency.

An adjustable bandwidth filter which can be varied from 300 KHz to 0.05 KHz processes the spectral signal before it is displayed. The pulse technique imposes certain limitations on this control which can best be explained by an example. In a typical situation, the pulse duration is 40 μs and the pulse is initiated 300 μs after the end of the previous pulse. The response time of the analyzer is controlled by the bandwidth frequency, e.g., for 300 KHz the response time is 10 μs and for 0.05 KHz it is 20 ms. This situation becomes clearer after realizing the analyzer needs more information to restrict the signal to a finer bandwidth. Since the second pulse occurs 0.3 ms after the first pulse, it would be undetectable for a bandwidth setting of 0.05 KHz. Therefore, in order to make absorption

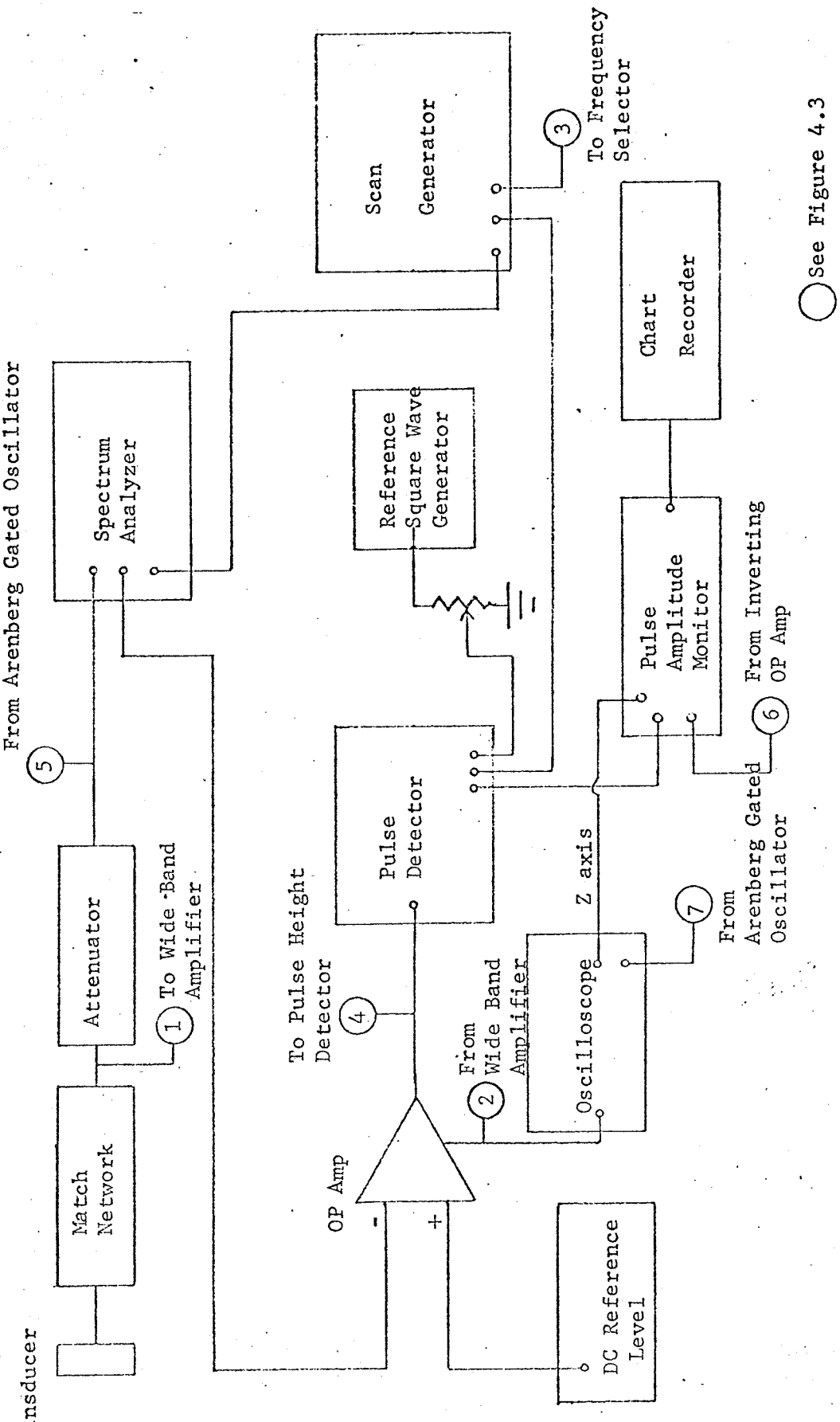

Figure 4.4. Block Diagram for the Receiving Pulse Circuitry.

measurements with the second pulse a lower limit is imposed on the bandwidth. When the setting is 30 KHz the pulse duration is approximately equal to the response time of the analyzer, and the top of the output pulse will be severely rounded. As the bandwidth frequency increases, the pulse will become flatter at the top. The bandwidth control setting can be used to advantage. The Arenberg does not produce a perfect RF pulse but creates a peak during the first few cycles. By carefully adjusting the bandwidth control the edges of the pulse can be rounded to eliminate this peak. All measurements were made with bandwidth control settingsof 100 KHz or 30 KHz.

The output pulse from the spectrum analyzer must be inverted before it is applied to the Model 1235 A Pulse Amplitude Monitor. An operational amplifier whose gain is two is used for this purpose. The function of the pulse amplitude monitor is to measure the amplitude in decibels of any pulse occurring during a fixed time interval, called the strobe. This strobe is synchronized with the transmitted pulse through an inverted synch pulse from the Arenberg gated amplifier. The strobe will occur simultaneously with the received pulse if its strobe delay vernier is properly adjusted. This operation can easily be accomplished after the pulse is displayed on an oscilloscope which is synched to the transmitter and is intensity modulated with the strobe.

The logarithmic output from the pulse height monitor is recorded by a model SR Sargent chart recorder, driven by a synchrous motor. Since the motors used in both measuring systems are synchronous, the motion of the chart recorder is directly proportional to the motion of the transducers. Therefore, the output from the recorder represents a plot of the natural logarithm of the received pulse's amplitude as a function of the transducer's position. Equations (4-3) and (4-6) give the total absorption and the excess absorption

for the high frequency system and the low frequency system, respectively. They show that when the transducers are moving, the recorder's output should be a straight line whose slope is equal to the absorption.

An interference signal is used to measure the acoustic velocity with the low frequency system. It is produced when the output pulse is amplified and mixed with the attenuated output from the oscillator exciting the Arenberg pulsed amplifier. The measurement is made by recording the position of the carriage with the micrometer slide, when the signals are out of phase. The carriage is then moved a distance which corresponds to "m" wavelengths and its position is again recorded when the signals are out of phase. The velocity is given by equation (4-5).

Velocity measurements for the high frequency system are made using a somewhat different procedure. The output voltage from the frequency selector is mixed with the pulse from the receiving transducer before entering the spectrum analyzer. The analyzer's output is inverted and then applied to a pulse height detector whose output is proportional to the pulse's maximum amplitude. As the piston moves through the cylinder, the phase between the signals applied to the analyzer will change. As a result the output voltage from the pulse height detector will vary from a minimum to a maximum and back to a minimum in the time needed for the piston to move an acoustic wavelength in the medium under investigation. The pulse height detector's signal is then applied to the time interval driver which produces a spike every time a minimum occurs. The time interval, Δt, between spikes is measured by the Systron Donner interval timer plug-in unit. In practice, Δt is averaged over 100 wavelengths and the piston is always moved between the same two points during a measurement. Equation (4-7) is then used to calculate the velocity.

The electronic instrumentation which has been described can be used for single frequency absorption measurements but additional circuitry is required for simultaneous multiple frequency measurements. To develop this kind of system, it is necessary to excite the transducer at a number of different harmonic frequencies. Since the Fourier series of a pulse has a rich frequency spectrum, the Model 179 Standard Pulse Generator was used to excite the transducer. By suitably adjusting the pulse width and its repetition rate, some of the transducer harmonics were excited. Although the amplitude of each harmonic decreased as the frequency increased, it was possible to keep their amplitude within 20 db from 3.9 MHz to 7.3 MHz. The quality of the signal was tested at the 7.3 MHz harmonic with the transducers stationary. It was assumed that the ultimate accuracy of the absorption measurement depended on the fluctuations of the chart recorder when the transducers were not moving. The test showed that the output from the recorder varied by 0.1 db for three minutes. The stability of the system using a single frequency oscillator as the input to the Arenberg gated amplifier was also tested. In this case, the variation of the chart recorder was only 0.03 db for three minutes. Based on this experiment, it was decided to abandon any system using multiple frequency excitation of the transducer.

The frequency selector whose circuit diagram is shown in Figure 4.5 was designed to allow single frequency excitation of the transducer for simultaneous multiple frequency measurements. It controls the frequency appearing at its output terminal from the scan ramp function of the spectrum analyzer. Four Schmitt triggers are connected to an equal number of coaxial relays through emitter followers. When the scan ramp is -5 volts, which corresponds to the analyzer's lowest frequency, all the relays are deenergized. As the scan voltage increases each Schmitt trigger in turn will

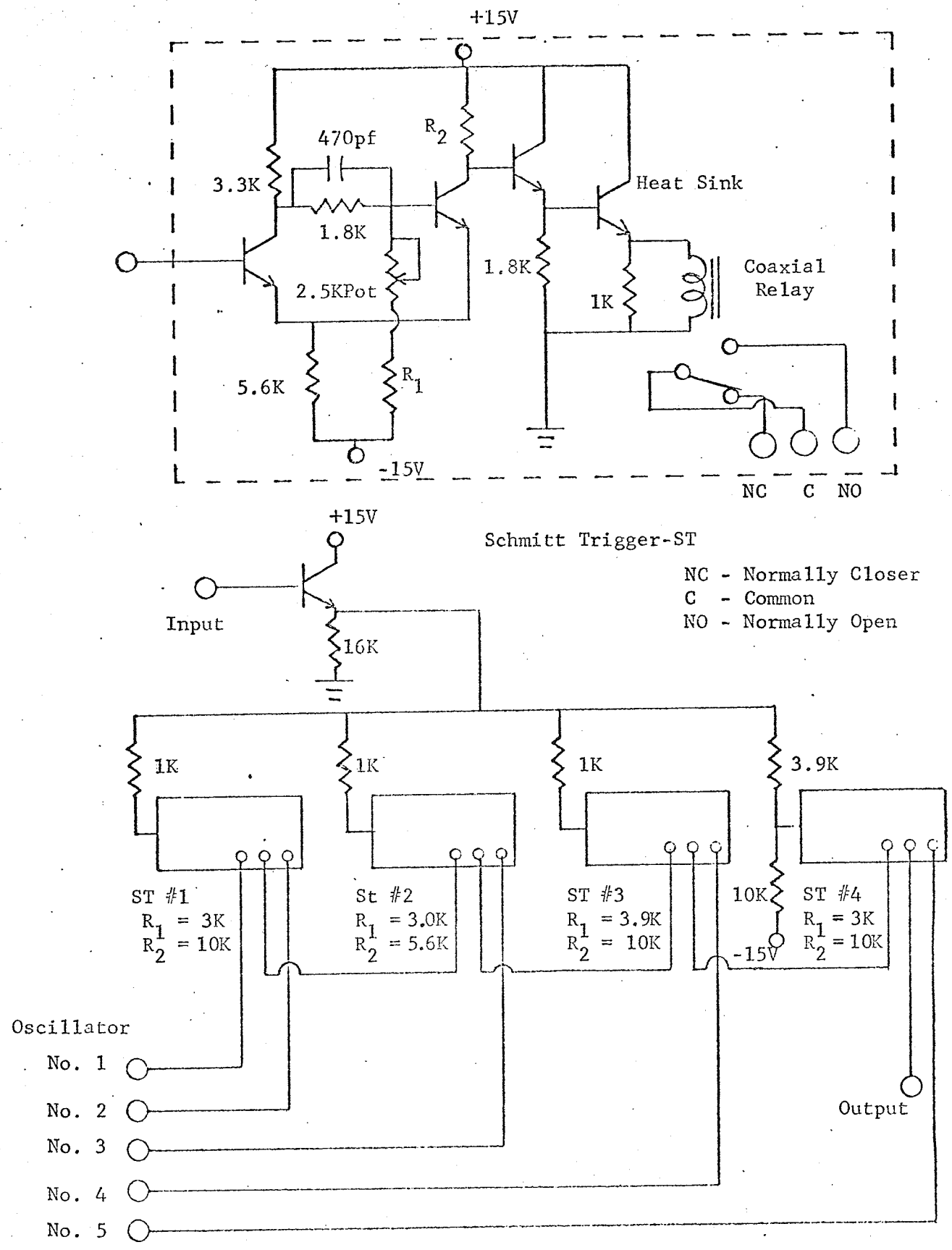

Figure 4.5. Frequency Selector Circuit Diagram.

energize a relay at a predetermined voltage which can be adjusted through an external potentiometer. By properly adjusting these pots, the correct harmonic frequency will excite the transducer when the analyzer is tuned to that frequency. The output from the analyzer is essentially zero except in a narrow frequency range which corresponds to the RF frequency of the transmitted pulse. Even at the slowest scan rate, the pulse amplitude monitor does not have enough time to make an accurate measurement.

This problem was dealt with using the variable rate scan generator shown in Figure 4.6. Two modes of operation are possible; one manual and the other automatic. The spectrum analyzer can be externally scanned with a 0-10 volt signal. Five volts must be subtracted from this signal before applying it to the frequency selector. A Zener diode was used for this purpose. The scan voltage for the manual mode is generated with a ten turn precision potentiometer. When the "manual-auto" switch is in the "auto" position, this voltage is generated by an integrating operational amplifier whose output voltage is proportional to the input voltage multiplied by the time it is applied. Normally the voltage drop across the n channel FET is small. Therefore, approximately -15 volts appears at the input to the integrator, and the sweep rate will be fast. If a +15 volts is applied to the gate of the FET, its impedance will be drastically increased and the voltage at the integrator will drop to a value determined by the scan rate potentiometer. This pot can be adjusted to control the "slow" sweep rate. When a complete scan has ended, the generator must be manually reset before another scan can be started.

Additional circuitry is needed to specify the time when the scan generator must be switched to its slow rate so that a measurement can be taken. The pulse detector shown in Figure 4.7 was designed for this purpose. It consisted of a zero crossing detector whose output was either +1.2 volts or

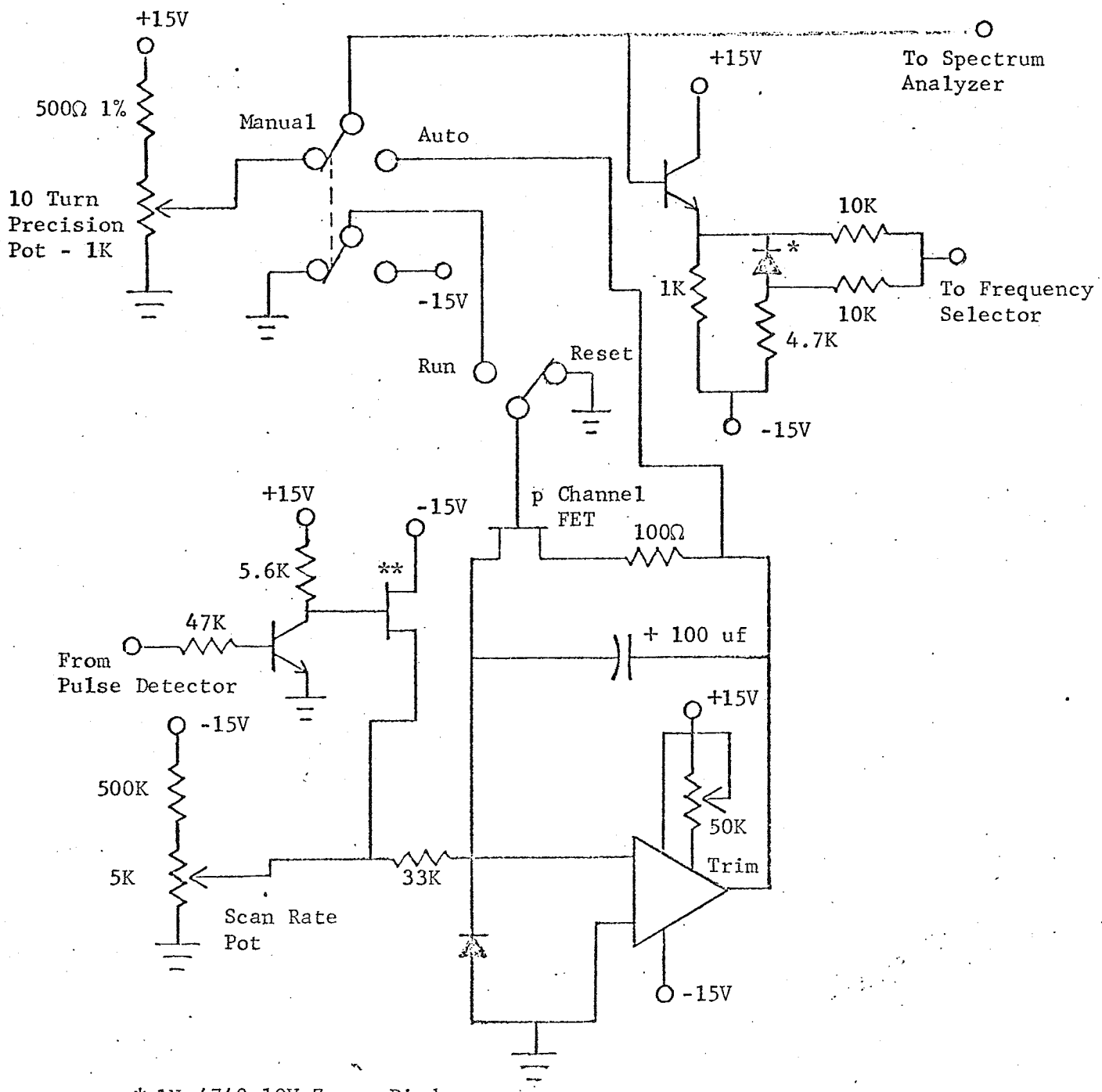

Figure 4.6 Scan Generator Circuit Diagram.

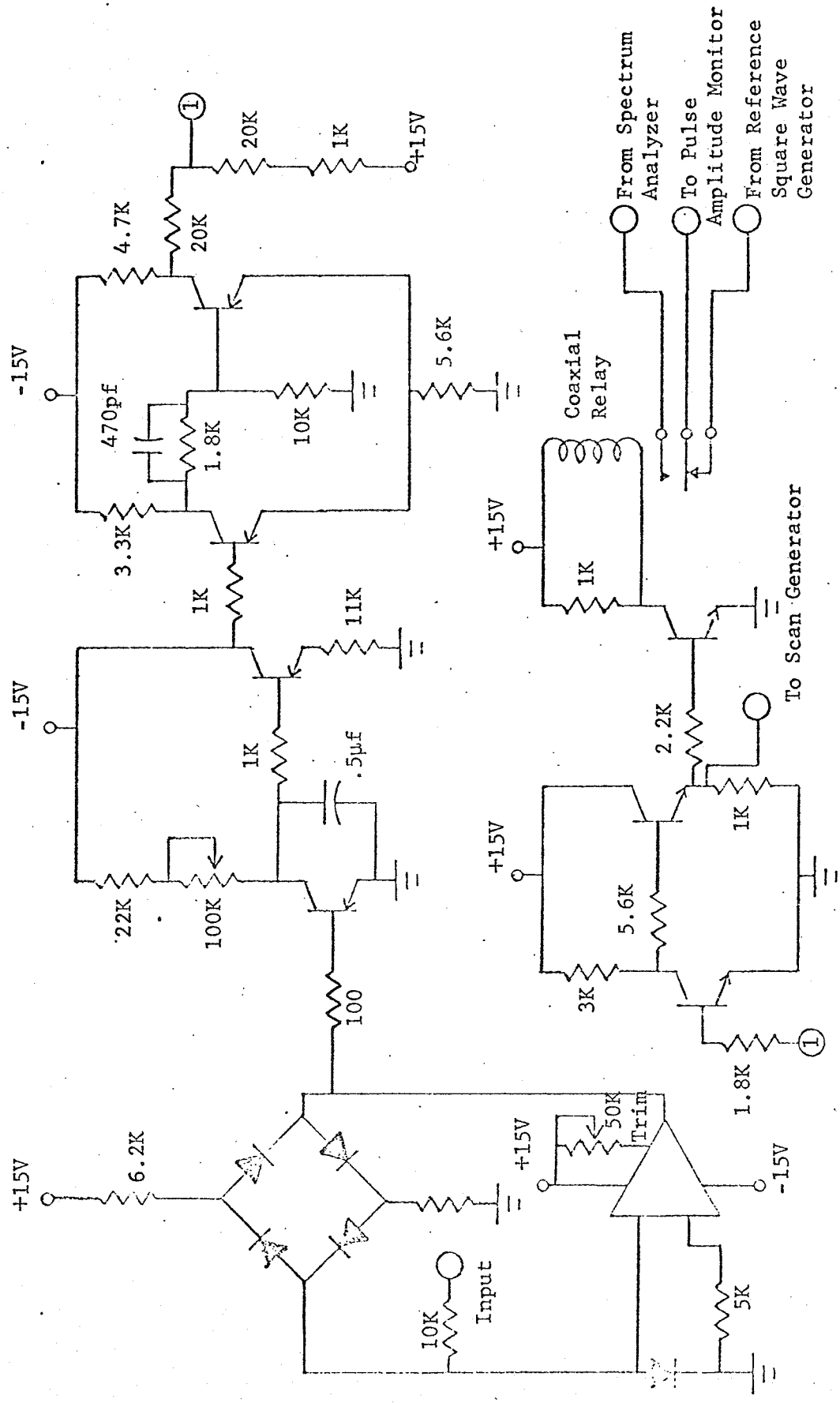

Figure 4.7. Pulse Detector Circuit Diagram.

-1.2 volts depending on the polarity of its input voltage. The sum of a negative DC reference voltage and the output pulse from the spectrum analyzer was used as its input. When the amplitude of the analyzer's pulse was greater than the reference voltage, the zero crossing detector changed polarity. Each time this occurred, a timing capacitor was discharged through a saturated transistor. The pulse from the spectrum analyzer appeared with the same repetition rate as the transmitted pulse. By adjusting the RC time constant, the capacitor voltage could be kept well below the supply voltage before it was again discharged by the next pulse. When the analyzer's output pulse dropped to zero, the capacitor would eventually charge to the supply voltage. By monitoring the voltage on this capacitor, it was possible to detect the presence of the output pulse. A Schmitt trigger was employed for this purpose. Since the pulse amplitude monitor would go off scale when the analyzer's pulse decreased in amplitude, a protective relay was added to the circuit. When the pulse detector sensed an output pulse from the spectrum analyzer, it was connected through the relay to the pulse amplitude monitor, otherwise a reference square wave generator of the proper amplitude was connected to the pulse amplitude monitor.

To summarize the operation of the instrumentation, the following events would occur when operated in the automatic mode. The scan control could sweep the frequency of the spectrum analyzer at the "fast" rate until the pulse detector switched it to the "slow" rate. This rate was adjusted so that the pulse amplitude monitor would have enough time to make an accurate measurement. This time was typically 15 sec. After the first transducer excitation frequency was passed, the scan control would again sweep at its "fast" rate. At this point the frequency selector would switch to the second oscillator to modulate the Arenberg gated oscillator. This cycle would be repeated for

all the remaining frequencies. To complete the cycle the scan generator was manually reset.

Three measuring techniques were developed using this equipment. When the scan control is operated in the manual mode, measurements are made at a single frequency. Measurements at a different frequency could be made by simply tuning the spectrum analyzer to the new frequency, if the oscillators connected to the frequency selector have been properly adjusted. This system is essentially the same as the one described by Kessler [1968] with the notable exception that a spectrum analyzer is used as the receiver.

Two automatic procedures were also developed. Both can be used for either the high or low frequency measuring system but will be explained specifically for the low frequency system. The carriage is positioned at one extreme and its microslide position is recorded. The scan control is placed in the automatic mode and a measurement is made. The carriage is moved and measurements are taken at two other positions. Data is taken at three points so that an average can be obtained. A typical measurement of this kind using four different frequencies could be completed in eight minutes. A comparable single frequency experiment would take twenty to thirty minutes. The main disadvantage of this technique is that the signal cannot be averaged over the entire transducer distance of travel as with the single frequency technique. Great care must be taken to insure that the signal is not affected by reflections from the window. This technique is referred to as the fixed transducer position method.

For the second automatic measuring technique, the transducers are kept in motion during the entire experiment, while the scan generator is allowed to run in the "auto" mode. The output from the chart recorder will be a series of peaks each representing a different frequency and whose maximum

amplitude should be a straight line whose slope is related to the absorption of the medium. In effect, the output at each frequency is sampled for different transducer positions. The transducer speed must be reduced by a factor of ten so that a sufficient number of samples can be taken to insure confidence in the measurement. The main disadvantage of this technique is the fact that it offers no reduction in the time required for an experiment compared to measurements made with the single frequency method.

Chapter 5

PRESENTATION OF DATA

The absorption of the medium differs from the values given by equations (4-3) and (4-6). These equations were derived assuming that the receiving transducer was excited with a uniform plane wave. Since the source transducer has a finite dimension, the assumption is poor and its actual radiation pattern at any point can be obtained by dividing it into elements and then superimposing the results for each element at the point. When this is done, the pressure amplitude will be seen to vary in a plane parallel to the face of the transducer; this effect resulting from diffraction. The relative pressure can be defined as the average pressure computed for a circular surface whose radius equals the radius of the source transducer. Diffraction effects will cause the relative pressure to vary as the distance from the source is changed. The Fresnel zone extends to a distance of a^2/λ from the source. Beyond this region the relative pressure is approximately proportional to the inverse of the distance from the source. Since the receiving transducer is piezoelectric, its output voltage will be proportional to the relative pressure incident upon its face. Variations in this voltage caused by diffraction effects can be accounted for by assuming an apparent absorption due to diffraction, α_d, which is given by

$$\alpha_d = \frac{\ell n\ \rho_1/\rho_2}{x_2 - x_1} \qquad (5\text{-}1)$$

where ρ_1 is the relative pressure at a distance x_1 from the source and ρ_2 is the relative pressure a distance x_2 from the source. The absorption of the medium will be the difference between the measured absorption and the absorption due to diffraction.

Del Grosso [1964] has calculated the relative pressure and tabulated results for different normalized transducer dimensions. His expression for the relative pressure contains Bessel functions whose arguments are given by

$$x = \pi/\lambda[(z^2 + 4a^2)^{1/2} - z] \qquad (5\text{-}2)$$

where λ is the wavelength of the acoustic wave, z is the distance separating the transducers and "a" is the radius of each transducer. This equation shows that diffraction corrections must be made when either the separation between the transducers changes during a measurement or when the wavelength of the medium changes.

Measurements made with the low frequency system must be corrected for diffraction effects because the acoustic wavelength of the reference liquid is not equal to the wavelength in the sample liquid. These corrections are made by assuming that the entire acoustic path length is in the reference liquid when the receiving transducer reaches its closest position to the window. The relative pressure is calculated by using the acoustic wavelength for the reference. A similar assumption is used when the carriage is moved to its other extreme position. The relative pressure is again calculated, this time using the wavelength of the sample. The absorption due to diffraction effects can be calculated from equation (5-1) and is then subtracted from the observed absorption. The result is the actual absorption of the medium. A sample calculation is shown in Table 5.1 to clarify all the steps in this procedure.

Diffraction corrections for measurements with the high frequency system can be a significant percentage of the measured absorption because the separation between the transducers changes with this system. A computer program whose block diagram is shown in Figure 5.1 was developed to calculate

Table 5.1

Sample Diffraction Correction for Low Frequency System

Radius of the transducer a		1.27 cm.
Frequency		5.0735 MHz
Measured absorption for 16.5% PEG @ 20.3°C		.01341 nep/cm.
c_x	- velocity of PEG	1560 m/s
c_w	- velocity of water	1483.2 m/s
λ_x	- wavelength in PEG	.03075
λ_w	- wavelength in water	.02923
$\frac{2\pi a}{\lambda_w}$	normalized dimension of the transducer	273
z	separation between transducers	12.0
$\frac{z\lambda_x}{a^2}$	normalized path length in PEG - x_1	.229
$\frac{z\lambda_w}{a^2}$	normalized path length in water - x_2	.217
ρ_2^*	relative pressure for x_2	898174
ρ_1^*	relative pressure for x_1	900357
$\ell n\ \rho_1/\rho_2$		.002428
α_d	absorption due to diffraction	.00020 nep/cm.
α	absorption of DNA	.01321 nep/cm.

*Used $2\pi a/\lambda_w = 100\pi$

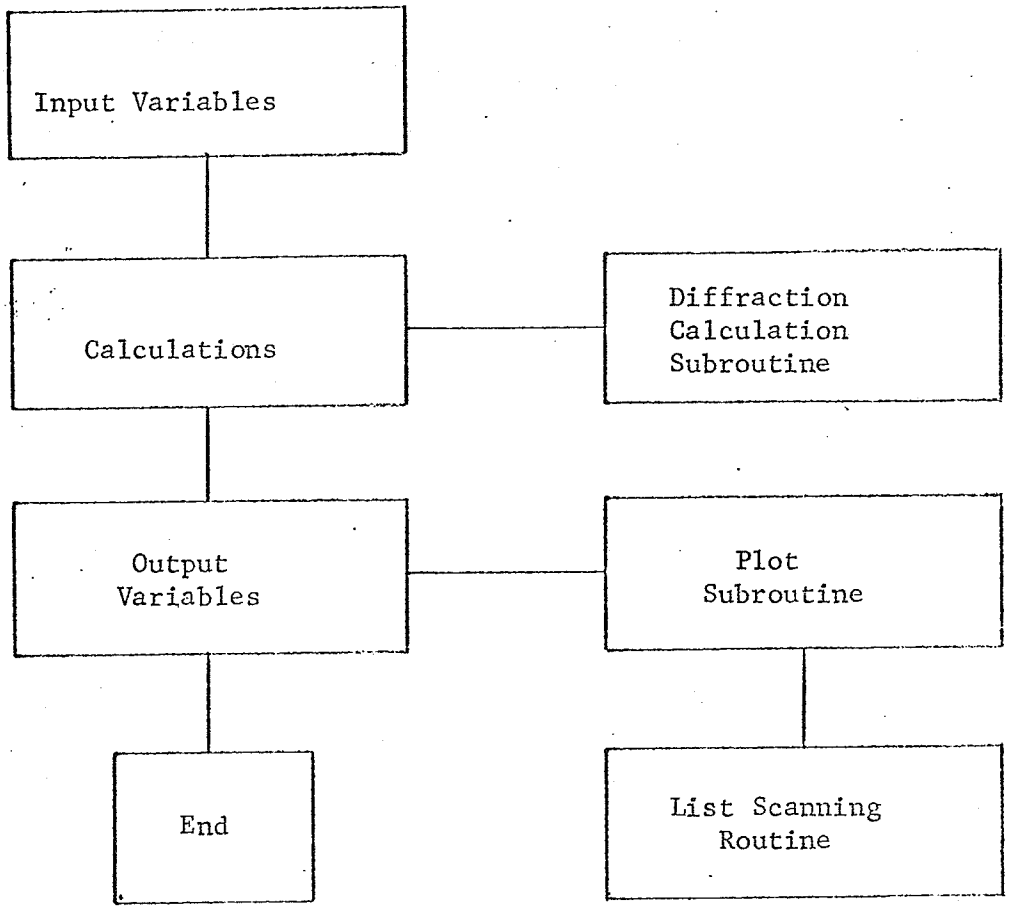

Figure 5.1. Block Diagram for the High Frequency System Diffraction Correction Program.

these diffraction corrections. A list of the program, a sample output and a series of plots are included in the Appendix. The program requires six input variables. The gear ratio, GR, must appear in column five of each data card. If it is zero or is missing, the program will stop. The remaining variables, pH, temperature, frequency, velocity, and the measured absorption are punched in five data formats which are each ten columns wide. If any of these variables are left blank or are zero, the program will replace them with their previous value. When calculating the relative pressure, a large argument approximation was used for the Bessel functions (Abramowitz and Stegun [1964]). This approximation is only valid for arguments greater than three. The relative pressure was compared to the tabulated results given by Del Grosso and they agreed to the fifth significant figure. The tabular output consists of all the input variables plus several calculated results. When the value of KA is greater than 30 the large argument approximation is valid. "RAW A" is the measured absorption while "DIFF A" is the absorption due to diffraction. The difference between them is labeled "Delta A" and it is the absorption of the sample. This absorption is divided by the square of the frequency and is tabulated under the column called the frequency free absorption, "FREQ FREE A." A list of the data was made in the program. By scanning this list plots of the frequency free absorption vs pH at a specific frequency were made.

Measurements were made with the low frequency system on a known liquid so that a comparison could be made. A solution of Polyethylene Glycol (PEG) was used as the test liquid. The PEG's molecular weight was 9700 and a solution whose concentration was 16.5% was used for the experiment. The results for this experiment are shown in Table 5.2 along with a comparison of these results with the data reported by Kessler [1968]. They are presented

Table 5.2

Comparison Measurements for the Low Frequency System

Polyethylene Glycol (PEG)				
Molecular weight				9700
Concentration (c)				16.5%
Temperature				20.3° C
Velocity				1560 m/sec.
Frequency (MHz) (f)	Absorption Coefficient (nepers/cm)	$\alpha/f^2 \cdot c$ measured	$\alpha/f^2 \cdot c$ known*	Difference (%)
5.07	.01321	311.0	308.2	0.9
7.33	.02400	270.7	256.8	5.4
9.58	.03656	245.5	230.8	6.4
11.8	.05101	222.2	208.0	6.8
			Average	4.9

*extrapolated values (Kessler [1968]).

by dividing the frequency free absorption by the concentration of the solution. This table shows that all measurements of PEG agreed with Kessler results to within 7.0%.

An acid titration experiment was performed on an SSC solution of Calf Thymus DNA whose concentration was 1.8%. Absorption measurements were made at four frequencies, viz., 5.07 MHz, 7.33 MHz, 9.58 MHz, and 11.8 MHz. The solution's temperature was 24.8° C $\pm$ 0.2° C and its pH was varied from 4.8 to 2.05. The results of this experiment are shown in Figure 5.2. The measurements for pH 2.05 are not directly comparable to the results for the rest of the experiment because a precipitate formed. All measurements were made using the manual single frequency technique.

Additional measurements were made with this solution to test the accuracy of the other measuring procedures. Comparisons of the results are presented in Table 5.3. A measurement was made at 5.07 MHz using the second pulse. When compared to the previous results it agreed within 10%. Both simultaneous multiple frequency measuring techniques were also tested for this solution at pH 2.05. The fixed transducer method was used to measure the absorption at 7.33 MHz, 9.58 MHz and 11.8 MHz, and the multiple frequency sampling technique was tested at 9.58 MHz and 11.8 MHz and compared to the values given in Figure 5.2. The results for both simultaneous multiple frequency methods show generally poor agreement with previous results for the same solution.

The high frequency system was tested at 10.2 MHz, 14.8 MHz, and 19.3 MHz in distilled water and then compared to the data reported by Pinkerton [1949]. The results shown in Table 5.4 agreed within 7.0%. Ultrasonic absorption measurements were made on Salmon Sperm DNA solutions at 10.2 MHz, 14.8 MHz and 19.3 MHz and at a concentration of 0.35%.

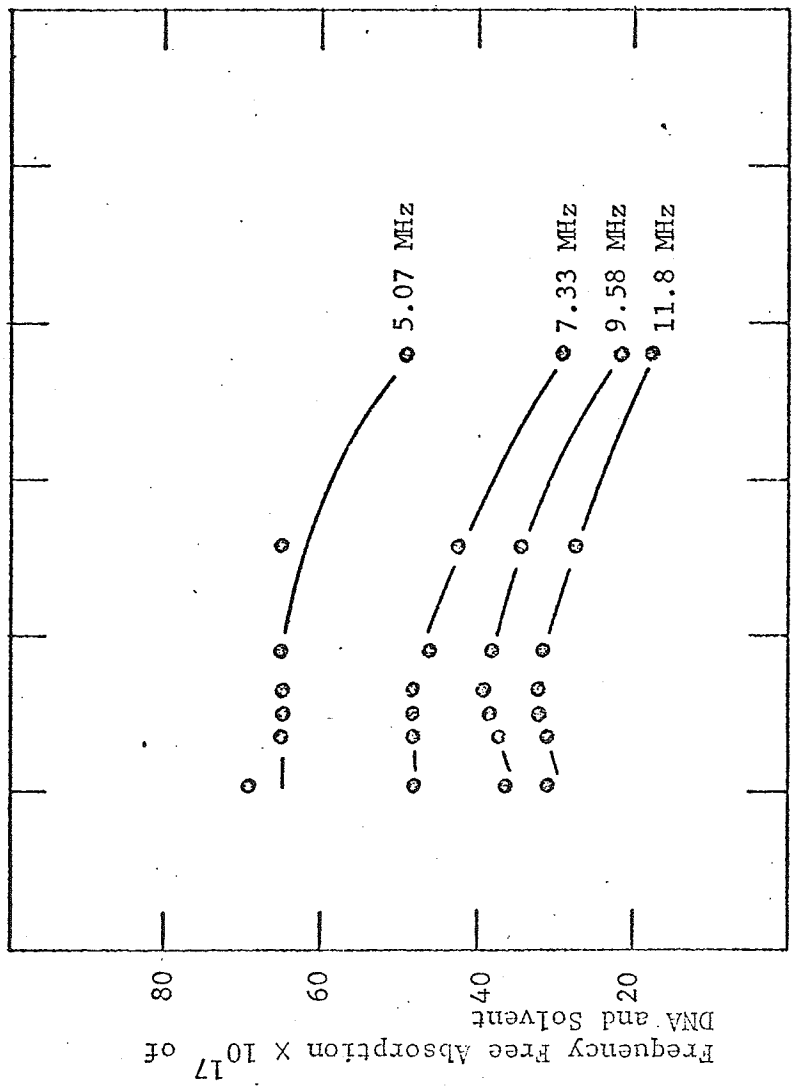

Figure 5.2. Ultrasonic Absorption Titration - Calf Thymus DNA.

Table 5.3

Comparison of Frequency Free Absorption for Three Different Measuring Techniques

Solution Calf Thymus DNA Concentration	1.8%
Temperature	24.8° C
pH	2.05

Frequency Free Absorption - $\alpha/f^2 \times 10^{17}$

Frequency (MHz)	SFT	SPT	FTPT	MFST
5.07	49.16*	44.15*	--	--
7.33	48.44	--	36.98	--
9.58	36.73	--	30.94	27.00
11.8	31.20	--	26.41	26.29

SFT -- Single frequency technique
SPT - Second pulse technique
FTPT - Fixed transducer position technique
MFST - Multiple frequency sampling technique

*ph - 4.80.

Table 5.4

Comparison Measurements for the High Frequency System

Distilled Water Temperature			25° C
Frequency (MHz)	Frequency Free Absorption $\times 10^{17}$		Difference (%)
	Measured	Known*	
10.22**	20.49	20.6	0.5
14.8	20.18	20.7	2.5
19.3**	19.2	20.6	6.8
		Average	3.3

*Pinkerton [1949].

**26.1° C.

An acid titration experiment was performed in which the pH was varied from 7.4 to 1.9. The diffraction correction program, shown in the Appendix, was used to calculate the results. They are plotted in the program as a function of the frequency-free absorption of the DNA and its solvent, and they show why the high frequency system must be used to measure this DNA. Since the frequency-free absorption of water is 20.6×10^{-17} at this temperature and the low frequency system measures the difference in absorption between the sample and water, it could not be used to measure solutions of this type of DNA. Considerable difficulty was encountered in making these measurements. The final procedure followed, consisting of stopping the piston for twenty minutes between runs. This period allowed the solution to reach temperature equilibrium. The piston was always traveling toward the bottom of the transducer while making a measurement and the circulation pumps were shut off for each run. These procedures caused the experiment to be prohibitively long.

Velocity measurements were also made for both DNA solutions so that the diffraction corrections could be made. Their results are shown in Table 5.5. The velocity of distilled water was measured at $25.7^{\circ}C$ with the high frequency system and the results agreed within 0.03% with the value reported by Greenspan and Tschiegg [1959]. The velocity of the Salmon Sperm DNA and the Calf Thymus DNA solutions were measured at a frequency of 11.8 MHz. Their velocity was assumed to be dispersionless.

Table 5.5

Velocity Measurements

Frequency (MHz)		Velocity (m/sec.)
14.8	Distilled water @ 25.7° C (measured)	1498.3
	Known for water (Greenspan and Tschiegg [1959])	1497.8
11.8	Calf Thymus DNA @ 24.1° C Concentration 1.8% pH 2.05	1511.6
14.8	Salmon Sperm DNA @ 25.2° C Concentration .35% pH -7.38	1510.3

Chapter 6

CONCLUSIONS

Electronic instrumentation was developed to allow the simultaneous multiple frequency measurements of the ultrasonic absorption coefficient in liquid media. The instrumentation was adapted to existing high and low frequency measuring systems. Both systems were tested using the single frequency measuring technique for liquids whose absorption was already known. The results show that the equipment was capable of absorption measurements within 7% of accepted values.

The absorption coefficient was determined for two aqueous solutions of DNA which differed in both concentration and molecular weight of the solute. Acid titration experiments were conducted to measure the effect of denaturation on the absorption. The same general trend was observed for both experiments; i.e., the absorption reached a peak in the same pH region in which the spectrophotometric measurements showed that the DNA was denaturing. Therefore a change in the ultrasonic absorption was detected as the structure of the DNA changed from its native form to its denatured state.

The initial pH of the Calf Thymus DNA was 4.8 which indicates it was partially denatured when the experiment began. Its denaturation can be attributed to the low ionic concentration of the solvent. If it is desirable to investigate the absorption in the pH range from 7.0 to 4.8 a higher concentration of SSC must be used as the solvent. Unfortunately, the acoustic velocity will increase as the salt concentration is increased. This may adversely affect the accuracy of the measurement by increasing the diffraction effect.

A great deal of difficulty was encountered in measuring the absorption of solutions containing Salmon Sperm DNA. Since the viscosity of DNA solutions

is related to the molecular weight of the solute molecules, the viscosity of the Salmon Spern DNA solution was expected to be high and this was qualitatively found to be true. Similar measuring difficulties were encountered when using high molecular weight PEG. These difficulties can be largely attributed to the high viscosity of the solutions.

Several different measuring techniques were tested using solutions containing the Calf Thymus DNA. The absorption coefficient was obtained by using the second pulse and comparison with previous data showed that this technique could be used to give reliable measurements. Both simultaneous multiple frequency techniques showed poor agreement to comparable measurements made with the single frequency method. All the results were low compared to accepted values indicating that the pulse amplitude monitor cannot yield an accurate reading during the time the scan generator allows for the measurement. These techniques could probably be made useful if a pulse amplitude monitor with a shorter time constant could be employed. The fixed transducer position method does have the advantage that it takes less time than a comparable single frequency experiment. Since its agreement improved as the frequency increased, this technique could be used for measurements at higher frequencies or for solutions having greater absorption coefficients.

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APPENDIX

DIFFRACTION CORRECTION PROGRAM FOR THE HIGH FREQUENCY SYSTEM