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Design and Applications of an On-Line Minicomputer System for Dispersive Infrared Spectrophotometry

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A significant portion of the spectrum-handling of modern Fourier transform infrared interferometer systems can be obtained from dispersive infrared spectrophotometers, by interfacing to a small minicomputer and the development of the necessary spectrum-handling software. This is described for a Perkin-Elmer 180 and a 16K Data General NOVA minicomputer. The necessary software is presented in flow diagrams, and the hardware interface is completely described. Example applications, including spectrum averaging and scaled subtraction, are also presented.

Infrared spectrophotometry has long had the reputation of being only semiquantitative. For this reason, some of the attention being lavished on Fourier transform infrared instrumentation is derived from misconceptions regarding conventional dispersive instrumentation. Recently (1) Laitinen described infrared spectroscopy as a discipline which had reached a period of senescence, a view which is not completely shared by the author. The dispersive instrument manufacturers are moving slowly into the world of today, an era of sophisticated, computer-based data acquisition and reduction. As they begin redesigning their 10-year old dispersive instrumentation with modern, high-speed detectors and associate them with low-cost mini- or microcomputers, the field will enjoy a second childhood. This paper describes what can be done by interfacing an on-line minicomputer to a dispersive infrared spectrophotometer.

The addition of digital data acquisition and reduction hardware to a reputedly irreproducible instrument may seem like a foolish proposition. Much of this "irreproducibility" is due to improper operation and misunderstanding of the normal trading rules regarding slit width, scan speed, etc. However, some of the simplest applications of digital data handling can significantly improve the quality of information from even the least expensive infrared instrumentation. For example, the capability to spectrum-average is a valuable technique for improving the signal-to-noise ratio (S/N) in regions of low transmittance. Averaging n spectra together produces an improvement in S/N equal to the square root of n. This technique has been employed to great advantage in Fourier transform infrared spectroscopy, where averages of 100 scans are often employed in order to increase the S/N for a high resolution spectrum. Additional advantages are available to the spectroscopist with some kind of computing capability, such as slit function deconvolution, mathematical smoothing, and separation of overlapping peaks.

Complex spectra, such as those of synthetic polymers, do not lend themselves to quantitative analysis of such things as plasticizers, fillers, etc., except where the substrate spectrum has no interfering absorption bands. With such complex spectra, though, it is often possible to obtain a reference sample which does not contain the analyte. The spectrum of

the reference material can then be stored as a baseline to be subtracted from spectra of samples containing the analyte. Using a computer, some fraction of this baseline can be subtracted from the spectrum of a subsequent sample. In this manner, the complex baseline is completely removed from consideration, and the spectroscopist can then focus on the difference spectrum for his analysis.

R. N. Jones, of the National Research Council of Canada, and A. Savitsky and R. W. Hannah of Perkin-Elmer Corporation began developing the use of computer-aided infrared data reduction in the early 1960's. Perkin-Elmer, at the time, offered an abscissa encoder for their 21-series infrared spectrophotometers that, when combined with a retransmitting potentiometer for the ordinate, could be employed as the fundamental components of a digital data acquisition system. Jones' research group at the NRC began acquiring digital data with such hardware on a Perkin-Elmer 621, and published a substantial amount of their software in NRC Bulletins 11–13 in 1968 and 1969 (2–4). The original NRC system was strictly an *off-line* system of infrared data handling, with no real-time information supplied by the computer to the operator. The advantages of an off-line system involve the ability to handle larger amounts of data, higher level programming, and having access to a wide variety of peripheral devices, while an on-line system offers instant turnaround to the user, real-time error detection, and the opportunity to smooth, ratio, plot, etc. while the sample is still in the spectrophotometer.

The on-line system described below is built around a Perkin-Elmer model 180 spectrophotometer, but is applicable to nearly all dispersive infrared instruments. It should be pointed out, however, that the Perkin-Elmer 180 is particularly well suited for the task at hand. The inherent digital design of the 180 makes some of the interfacing simpler, but the concepts are the same for any other instrument outfitted with 1) an abscissa interval marker and 2) a retransmitting potentiometer for the ordinate. In fact, in an earlier paper (5) we described an equivalent interface for an inexpensive ($3500) Hilger-Watts H-1200 spectrophotometer, which is quite adequate for most routine infrared data acquisition.

The investment required for the interface between an infrared spectrophotometer and the I/O bus of a minicomputer is surprisingly small. In the case of the Hilger-Watts H-1200, the total cost of the electronic components in the interface was under $450; for the 180 interface, the cost was on the order of $2500. For laboratories that already possess small computers, there is no additional cost beyond that of the interface.

INTERFACE DESIGN

Perkin-Elmer offers a "standard interface" for the 180, which outputs a 13-character serial ASCII string at each data interval. The ordinate is transmitted with a precision of 1 part in 12 000, and the abscissa to 0.01 cm^{-1}. Figure 1 illustrates the timing required for the computer-generated "accept" signal to interact properly with the "ready" signal from the standard interface.

Since the putput of the standard interface is serial ASCII, in TTL logic, there is no problem in making the necessary connections to the I/O bus of a Data General NOVA. For a

different minicomputer, the interface network will be similar, so the following section is intended to be general enough to allow its application to any external computer.

The NOVA has a 16-bit word length and four hardware accumulators, two of which can be used as index registers for indirect addressing. It also has a hardware 15-bit program counter to keep track of the address of the current instruction. Any memory word can be used as storage for instructions, other addresses, or data. Memory words are byte addressable as well, enabling the user to store data, such as ASCII or 8-bit data, as pairs of 8-bit bytes, thus doubling the usual storage capacity.

Input and output are handled on the I/O bus, using either programmed transfers or interrupts. Programmed transfers are adequate for a spectrometer, since it is not able to hold up data while another device is operating. If the spectrometer is serviced by an interrupt rotine, it should be given top priority. We have compromised by turning over total control to the 180 while it is scanning, while driving the cassette tapes and teletype on an interrupt basis.

Prewired logic on the general-purpose interface board includes the circuitry that connects the interface to the data lines and I/O transfer signals on the I/O bus, logic networks for passing the interrupt and data channel request signals through the interface, and a device selection logic network that allows the interface to recognize its device code. The device code is set by the user by putting in the appropriate jumpers. Prewired I/O transfer signals include BUSY, DONE, START, CLEAR, (special) I/O PULSE, DATA IN A, B, or C, DATA OUT A, B, or C, INTERRUPT REQUEST, and INTERRUPT DISABLE.

The input logic is shown in Figure 2. In Figure 2, the data lines of the I/O bus are labeled DATA XX. Data can be strobed onto the I/O bus only by issuing the software command DIA, followed by the appropriate device code. The logic "1" coming from the OR gate at the right of Figure 2 triggers the NAND gates of DATA 9 through DATA 15, putting the seven-bit ASCII character directly on the bus. The interposition of a buffer register on the interface board, between the input data lines and the I/O bus, is not shown, but has been found to be an improvement over the original input design. As shown in Figure 1, the data lines from the spectrometer are held valid until after the "accept" signal is received.

The "ready" and "accept" signal interfacing is shown in Figure 3. In the middle of Figure 3, the top two flip-flops constitute the input interfacing logic. Data general includes the DONE flip-flop on the basic interface board. This flip-flop is clocked by the spectrometer's "ready" signal, which in combination with a "1" from the device select network (indicating that the software is waiting for data), sets the active state of $\overline{SELD}$. The software then resets the DONE flip-flop and generates an ACCEPT signal by clocking the ACCEPT flip-flop with the special I/O PULSE. This allows the computer time to take in the data, after which ACCEPT is reset to "∅" by the action of a DOB command. (Since only one device is connected to this board, the DOB, DOC, DIB, and DIC commands address non-existent buffers and can be used as extra software commands to the spectrometer).

The output circuitry is also shown in Figures 2 and 3. Figure 2 illustrates the connection of the digital-to-analog converter to the I/O bus. Binary (scaled) values are continuously varying on the I/O bus, and the DOA signal is used to strobe the contents of $\overline{DATA\ 6}$ through $\overline{DATA\ 15}$ into the buffer of the DAC. Upon conversion, the DAC output (0 to −10 V) is available at the analog output. An operational amplifier is used to invert the signal, and a voltage divider scales it down to 0 to 2.3 V for insertion into the 180 recorder's ordinate input.

On Figure 3, we show another flip-flop used as an oscillator. The output of the bottom flip-flop in Figure 3 is a software-controlled pulse generator. Using the $\overline{DIC}$ and $\overline{DOC}$ commands
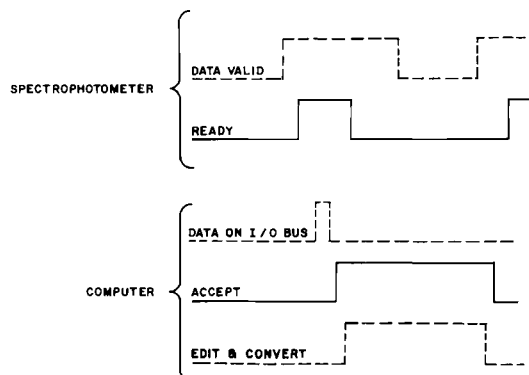

Figure 1. Timing diagram for digital signals between spectrometer and computer

to set and reset the flip-flop, the pulse rate is adjustable by software control, and is directly connected to the stepping motor driver on the instrument's chart recorder.

Standard cassettes for a NOVA system will hold about 55 000 16-bit words of data. However, even if one operates the system under DGC's Stand-alone Operating System (SOS) with the writing of interrecord gaps under a predetermined format, the practical capacity of a cassette is about 44 000 words, or 22 000 floating point numbers. Storing spectra as ordinate values only, in floating point number format, preceding each spectrum with an ASCII title line, one can store five 4096-point spectra per cassette.

Other peripheral devices, such as large-format magnetic tape, fixed or moving head disks, or even the new "floppy" disks, may be preferable to cassettes. The capital investment required, though, is usually greater than that for cassettes. One could also present a good argument for a CRT-style terminal and a high-speed paper tape reader instead of a standard teletype, but the difference in cost is substantial.

Adjusting Live Zero and 100% T Counters. The Model 180 uses an electronic counter, which counts from zero to a maximum of 12 000, to represent the ratio of the sample beam intensity to the reference beam intensity (I/I_o). When the sample beam shutter is closed, a minimum count, set between 50 and 200 counts, is produced. The minimum count provides for a "live zero," so that very low transmittances can be recorded with about 1% precision. The zero count is adjusted via a trimpot on the analog filter pc board. The full count trimpot on the same pc board can be used to adjust the 100% T count close to 10 000, provided that the sample and reference beams have been properly phased. As a rule, neither the live zero count nor the full scale count match their nominal values of 100 and 10 000, respectively. For this reason, it is necessary to have this routine in order to adjust software counters to represent the actual output of the spectrometer. This is the only way in which one can be certain of matching results over long periods of time, over which time the source may be changed, pc boards may be replaced or adjusted, external temperatures may change, and so on. A straightforward logic flow is employed which averages 100 points with the sample shutter closed, then 100 points with both beams empty, to get representative values for the zero and 100% T counters.

Spectrophotometer Data Acquisition. Entering the data acquisition routine generates requests for the starting wavenumber (CMST), the data interval (INCRE), the number of points to be acquired (NPTS), whether or not averaging is desired, and if so, the number of spectra to be averaged (AVGN). The operator starts the spectrophotometer scan after all of the instrument settings have been made.

According to Savitsky and Hannah (6, 7), a model 180 will not accurately respond faster than about five data intervals per second. In addition, our smoothing routine requires about
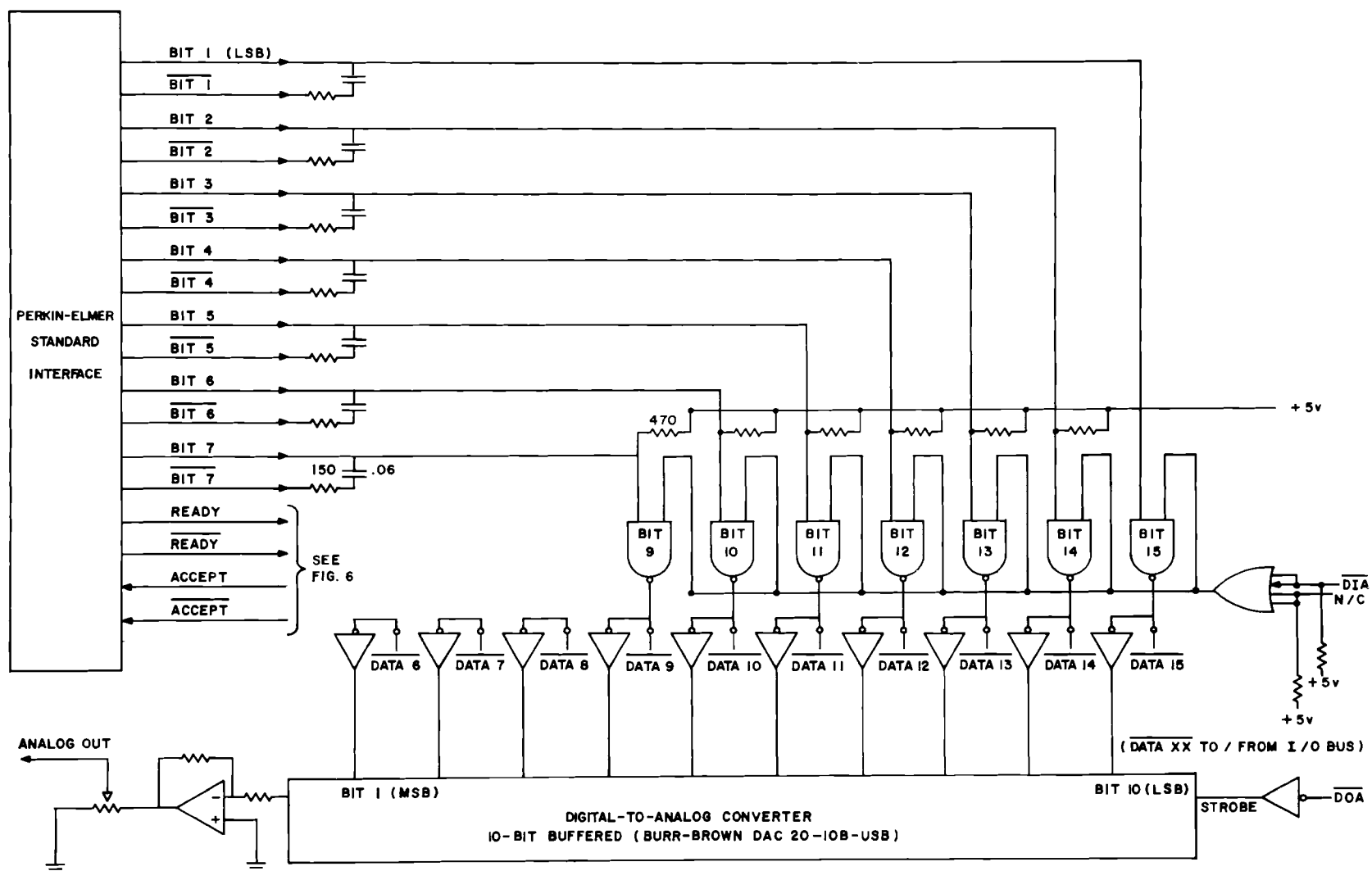
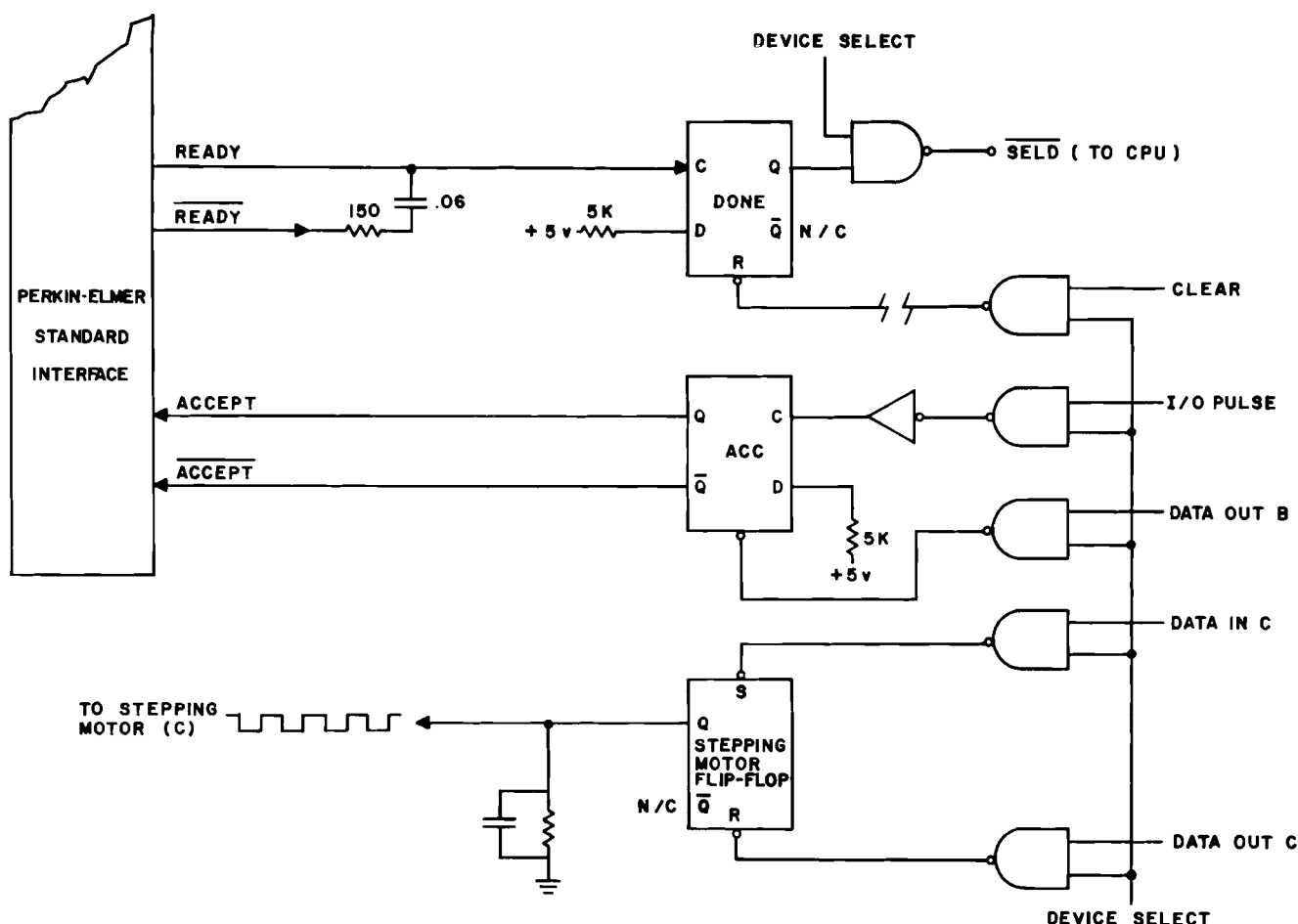**Figure 2.** Input circuitry for ASCII data from spectrometer to I/O bus, and analog output to recorder pen

Figure 3. Ready and accept logic circuitry, and stepping motor pulse generator

a 21 point half width (FWHM) to avoid distorting the top of a peak. These two restrictions serve to guide the operator in setting the adjustable parameters of the spectrometer. For the narrow absorption bands of, for example, a No. 2 fuel oil, a data interval of 0.2 or 0.5 cm^{-1} may be necessary, along with a modest scanning speed (1 to 2.5 cm^{-1}/s), and a narrow slit width. For broader absorption bands, for instance those of large proteins, a data interval of 1 or 2 cm^{-1} is sufficient, larger slit widths can be employed, as well as higher scanning speeds. Naturally, the S/N is improved at larger slit openings, thus reducing the need to average spectra in those instances. There does not appear to be any point in scanning at slower speeds than 5 data intervals per second, since no improvement in S/N should result. The S/N is determined by the absolute attenuation of the sample and reference beams, and the gain and slit settings of the spectrophotometer. When the S/N is low, enhancement can be obtained through spectrum averaging and/or smoothing. The operator of the computer–spectrometer system must be familiar enough with the available tradeoffs, both in the spectrometer and in the data handling, to be able to take full advantage of the capabilities of the system.

Figure 4 illustrates the flow chart for the acquisition of abscissa and ordinate data from the spectrophotometer. Using a Floating Point Interpreter, the ASCII character strings are converted to floating point numbers.

When the ASCII-to-floating point conversion routine encounters a non-digit ASCII character, the conversion routine terminates. In the event of a bad conversion, or a non-digit ASCII character that was not preceded by a digit, or an over-or underflow, checkwords are provided for "conversion OK" and "over-or underflow." Checking these words, as well as clearing

them each time a conversion is called for, must be done by the user.

The single most important feature of the spectrometer data acquisition logic, shown in Figure 4, is the section which edits incoming data on-the-fly. The editing routine begins at step 8 of Figure 4. Basically, it consists of a careful test of the abscissa to determine whether it matches the anticipated abscissa ($\pm \frac{1}{10}$ of the data interval), is a grating change, or is a bad data point. The abscissa is output as a six-character ASCII string, representing the abscissa to 0.01 cm^{-1}. The anticipated abscissa, CMST, is compared to the value just received, ν, using double-precision binary subtraction rather than floating point arithmetic to eliminate round-off errors. A precise agreement causes the exit to step 10 at GOOD. Any difference between CMST and ν goes into TEMP0, where its absolute value is tested against EPSI (=INCRE/10). If ν was within EPSI of CMST, the data are allowed. If not, TEMP0 is rechecked to see if it is greater than CMST, and if so, the data are rejected and a new set of data is requested. If not, it is tested to see if ν is only one data interval ($\pm 10\%$) less than CMST. If so, a grating change has just been passed and the data are accepted, setting SW1 in addition. The 180, for some obscure reason, does not transmit regular data at a grating change, but skips one data interval, thus SW1 is set in order to store the subsequent datum twice. A precaution to the user is to avoid setting up a scan so that a grating change is the last point in the scan. If this is done, the spectrometer must be allowed to pass through the grating change past the next data interval in order to acquire the final point. Other programmers may wish to use the ASCII ⟨35⟩ output from the standard interface as an indicator of a grating change. Electromechanical, low-speed, abscissa encoders can transmit substantial numbers of meaningless
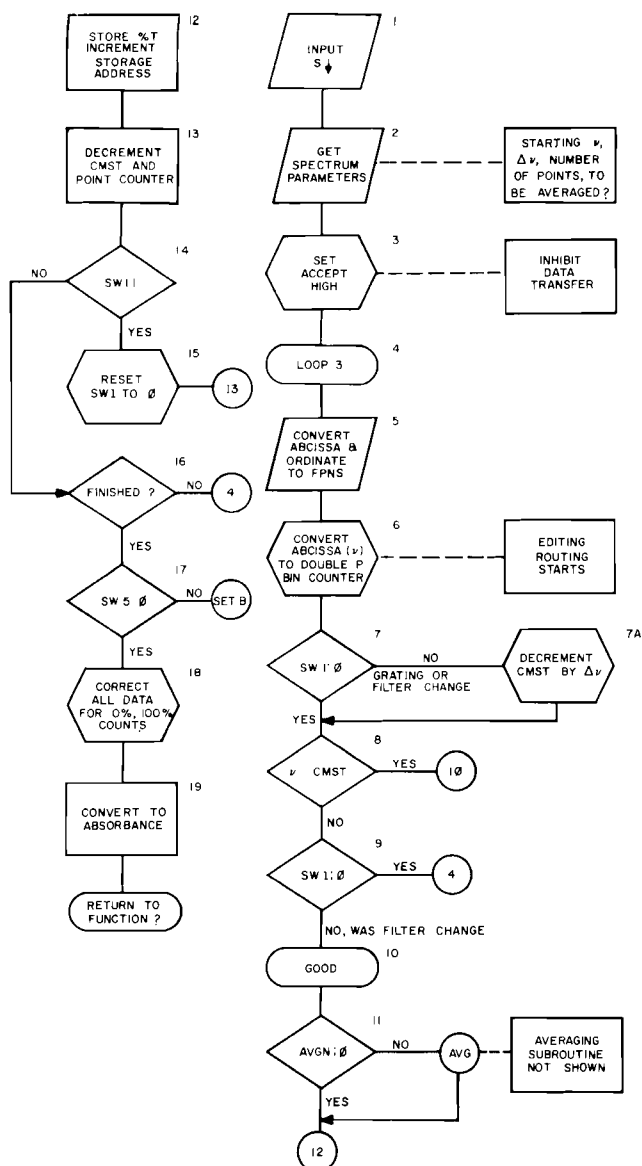
Figure 4. Flow chart. Spectrometer data acquisition

data if they become dirty or scratched. The editing routine described above and shown in Figure 4 is an absolute necessity to ensure proper registry when plotting, ratioing, or averaging spectra.

The remainder of the flow chart shown in Figure 4 is straightforward. Once the abscissa is determined to be good, the routine decrements CMST by one data interval and tests AVGN to see if averaging was requested. If averaging is to take place, the subroutine AVG (not shown in Figure 4) takes the floating point ordinate, divides it by AVGN (the number of spectra being averaged), and adds the result to the previously stored value for the same ν. As the data are entered, they are stored as $\%T$ counts (between zero and 12 000 counts) in floating point notation. This enables the computer to take data at very high speeds, much faster, in fact, than the model 180 can scan. At the end of the spectrum (or spectra, if averaging), the entire spectrum is corrected for the live zero, then converted to absolute absorbances (ranging from about -0.05 to about 2 absorbance units).

Plotting or Smoothing Spectrum in Memory. Figure 5 illustrates the logic flow followed in response to an "smooth" or a "plot" response. The smoothing routine is based on Savitsky and Golay's (8) moving, 21-point quartic smooth [as modified (9), and discussed in later papers (6, 7)]. The 21-point smooth gives a S/N improvement of about a factor of 2
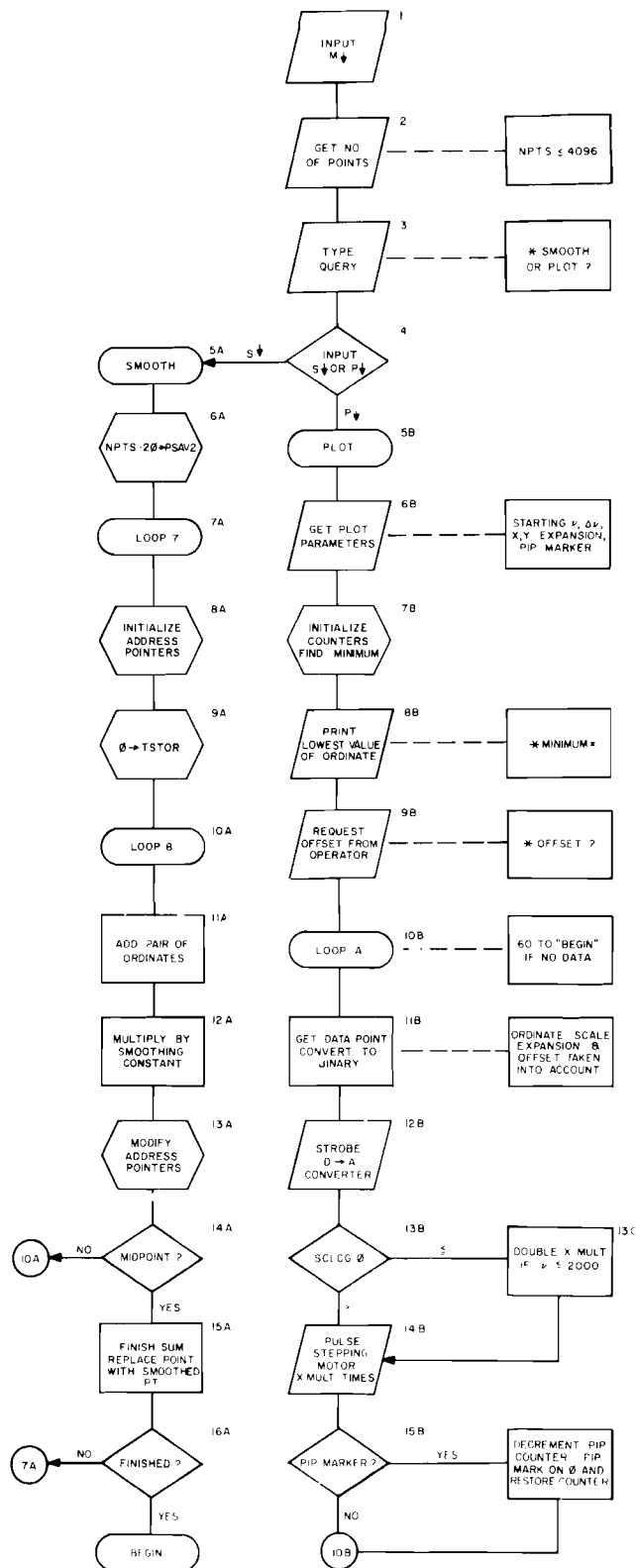

Figure 5. Smoothing or plotting data in memory

(6, 7), and requires that certain precautions be followed when recording the spectrum. First, the half-width (FWHM) of the narrowest peak of nterest should not be less than 21 data intervals; otherwise noticeable degradation in the peak height will occur upon smoothing. Second, adjacent peaks which are to remain separated after the smooth must have their peak maxima located at least 21 data intervals apart. When these two conditions cannot be met, the alternative available to the operator is to average four spectra together, with an attendent improvement in S/N of 2 $(= \sqrt{\text{AVGN}})$. In setting up the ν

limits, one must remember that, in the 21-point smoothing operation, the first and last ten points of the spectrum are not modified in any way.

The course of action taken after a "plot" response is shown in Figure 5. The computer requests sufficient information to set up the plot, including the starting wavenumber, the data interval, abscissa and ordinate expansion factors (the abscissa expansion factors must be multiples of $\frac{1}{2}$ and the ordinate expansion factor may be any positive number from zero to 10^{64}), whether or not a pip marker is desired, and if so, the interval (in points) between pip marks. Next, the computer searches the stored spectrum, finds the minimum absorbance value, prints it, and is prepared to plot the plotted spectrum. Before plotting, however, the operator is allowed to enter an OFFSET either to displace the spectrum so that the plot will display absolute absorbance values, or to vertically offset the spectrum any amount desired. Because of a plotting scale change at 2000 cm^{-1}, the computer subtracts 2000 cm^{-1} from the starting frequency in order to set up a binary counter, SCLCG, which will automatically double the number of stepping motor pulses per ordinate when 2000 cm^{-1} is reached on the plot (plotted from high wavenumbers to low). Each ordinate, varying between about 0 and 2 absorbance units, is first adjusted for the minimum and offset; i.e., plotted ORDINATE = ORDINATE−MINIMUM+OFFSET. This system employs a 10-bit D → A converter, and the unexpanded ordinate scale of the 180 is from 0 to 1.5 absorbance units, so each ordinate is multiplied by a scale factor of 682.67 before it is converted to a truncated binary integer, and the most significant 10 bits are strobed into the buffer of the DAC. The pip marks are produced by simply adding ten percent of full scale to the contents of the DAC buffer, without moving along the X-axis.

Write or Read a Cassette File. Data General's Stand-alone Operating System (SOS) provides the user with simple I/O calls to standard peripheral devices. Calls such as .RDL, .WRL, .RDS, and .WRS, for read a line, write a line, read sequential, and write sequential, make it easy to move data between cassette files and core memory. Figure 6 illustrates the read and write flow diagrams. Each read or write command requires a byte counter and a byte pointer, which are passed to SOS in the hardware accumulators AC0 and AC1.

Each data file produced by the main program begins with an ASCII title line, which is typed in by the operator before the data are transferred to the tape. The title line should contain such information as the starting wavenumber, data interval, number of points, and some kind of sample identification. This makes it easy for multiple users to keep track of their data files over long periods of time.

When a file is to be input following a "Read" response, the title is typed on the teletype before the computer requests additional information from the operator. Data are transferred, both in read and write mode, as a single block of 4· NPTS bytes. Ordinate values are stored sequentially as floating point numbers, each occupying two words. ASCII strings are stored one character to a byte, two bytes to a word. As mentioned above, about 44 000 words can be stored per tape cassette.

Spectrum Subtraction. A flow chart is unnecessary for this simple but powerful routine. The spectrum which is the minuend is placed on one cassette drive and the baseline spectrum goes on another. The computer requests the file numbers for the two sets of data, then reads in the entire sample spectrum. A scale factor is then requested from the operator, to be used in a scaled subtraction. Baseline ordinate values are then read in one at a time, multiplied by the scale factor, then subtracted from the minuend stored in core. The differences replace the spectrum in memory, one point at a time. Since SOS uses a software buffer to transfer bytes in
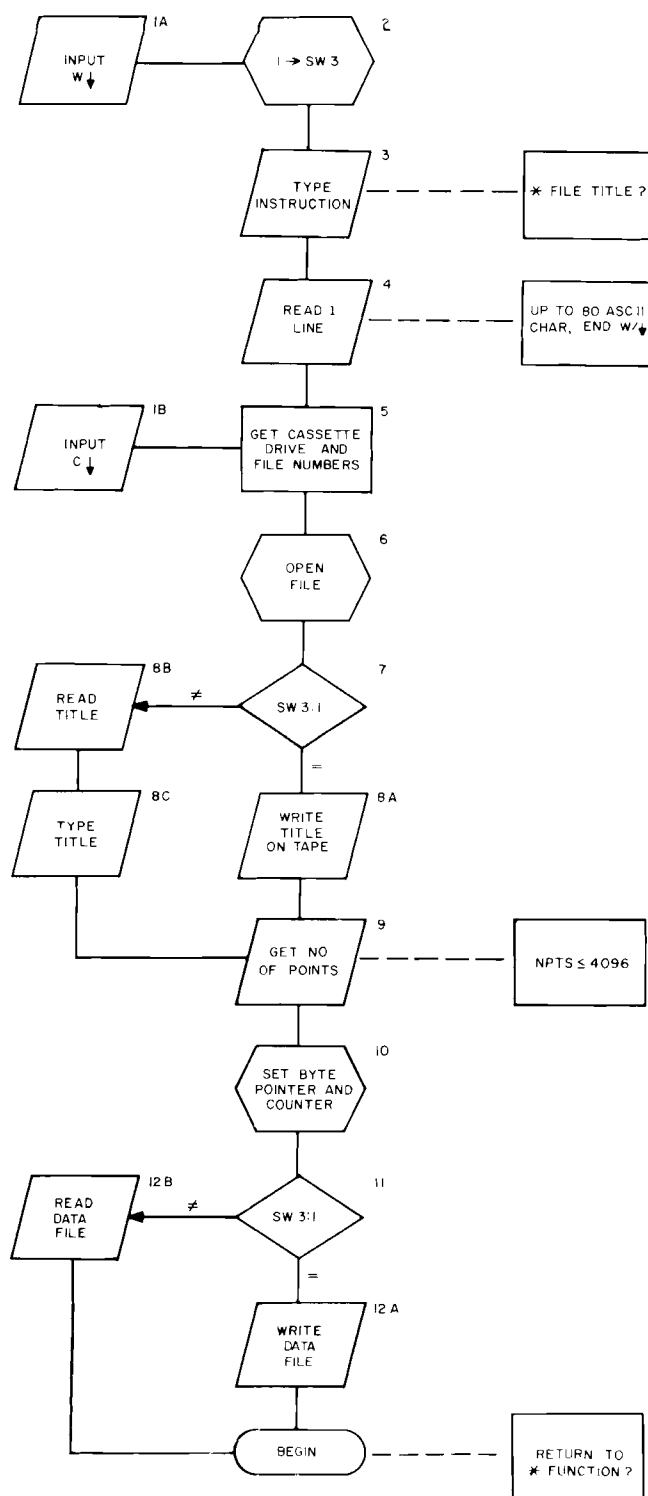

Figure 6. Write or read a cassette tape file

blocks of 510 from a cassette tape, the operation of reading four bytes at a time does not cause jerky tape motion. The above routine conserves space by not requiring storage for more than one spectrum in core.

Type Points on Teletype. This is a simple function used primarily for testing purposes. It could be used to produce paper tapes of spectra, but they are not very convenient to handle. To conserve paper, this subroutine simply formats the ordinate data into ±XXX.XXXXX strings, five to a line, on the teletype. The numbers of points to be typed are selected by the operator, always started with the first one in memory.

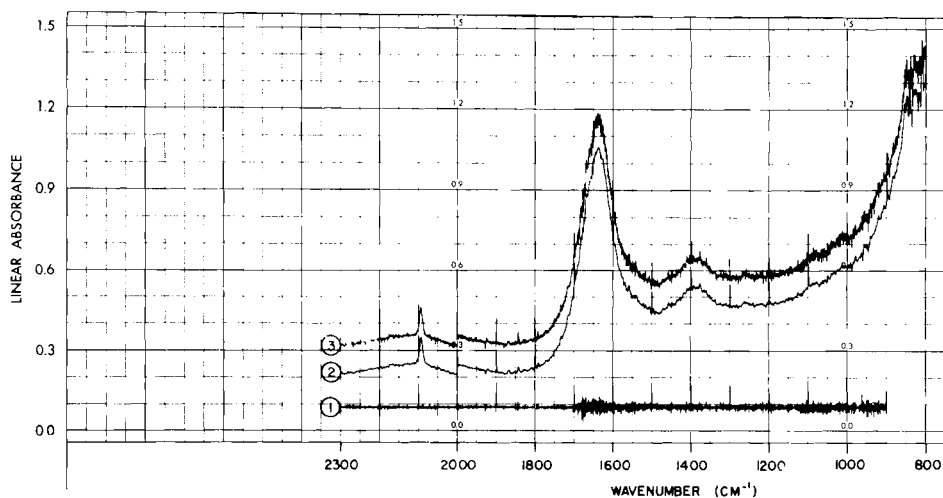The main program, described above, requires only 178 page

Figure 7. Example of a 21-point smooth of a spectrum of aqueous CO_3^{2-} and CN^-. Bottom spectrum is difference between raw and smoothed data, showing level of noise reduction obtained
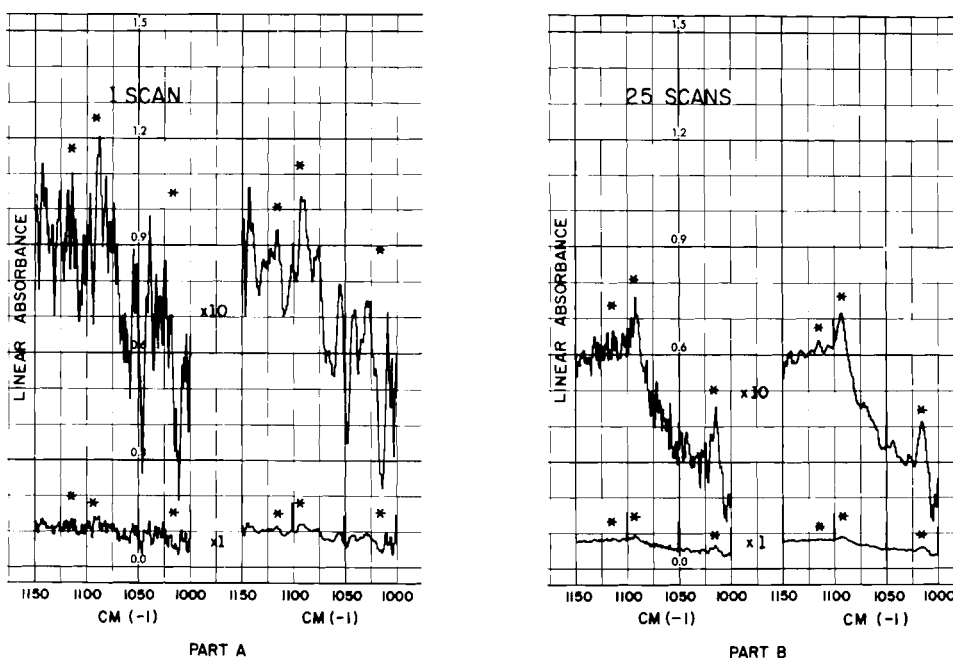

Figure 8. Signal-to-noise enhancement through a combination of spectrum-averaging and smoothing. Part A illustrates raw (left) and smoothed (right) spectra for a single scan of 1 μg of p,p'-DDT on a KRS-5 micro-ATR prism. Part B illustrates the advantages obtained by averaging 25 spectra of the sample, a process which took less than 25 min, at 4 cm^{-1}/s. The asterisks indicate the absorption bands expected at 1116, 1094, and 1016 cm^{-1}. Ordinate values are in linear absorbance $\times$ 10

zero relocatable and 1409 normally relocatable 16-bit words of core memory. An additional 8192 words is set aside for storage of a 4096 point spectrum (as floating point numbers, each requiring 2 words). The use of floating point numbers requires loading the Floating Point Interpreter, a relocatable binary set of subroutines which occupies an additional 1960 core locations. The program runs under the control of DGC's Stand-alone Operating System (SOS), which handles the cassette tape drives and the teletype on an interrupt-driven basis. The total set of programs required for operation includes the cassette driver, the teletype driver, the SOS library, the Floating Point Interpreter, and the main program. These programs occupy 222 of the 256 page zero locations and 14 131 of the total 16 384 core locations (8192 assigned to spectrum storage). The machine language assembled programs are stored as a core image ("savefile") on a single cassette file, and can be loaded in about 30 seconds by the core image loader. (The core image loader resides permanently in high core and is not destroyed by any of the programs).

In addition to the operations carried out by the main program, individual users have written data reduction programs, including peak locating, integrating, peak separation, slit function deconvolution, etc., in BASIC. DGC's Extended BASIC is also loaded from a cassette savefile in a matter of seconds, and the individual's programs can be loaded under SOS+Extended BASIC by the BASIC keyboard command LOAD "CTX:YY". Individual programs which overrun the capacity of the 16K memory can be linked and swapped using the BASIC "CHAIN" instruction. Thus, one can run programs of any length under SOS+Extended BASIC, even though the SOS library and the BASIC interpreter take up nearly 11 500 words of core. Data files written by the main program are in floating point format, with the first record in each file containing an ASCII title line. Both the title line and the data file are compatible with BASIC I/O logic. The combination of the smaller, more efficient main program, with the high level programming capabilities of DGC's Extended BASIC, makes the University of Miami computer–spectrometer system much
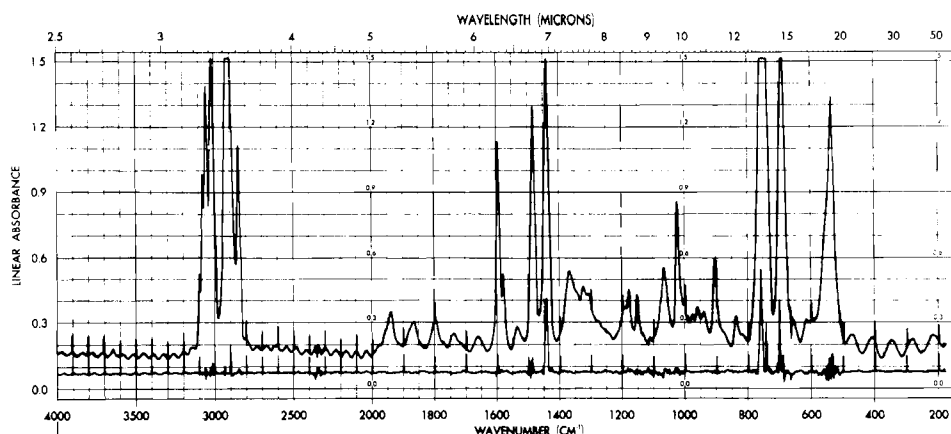
Figure 9. The polystyrene spectrum shown is really two superimposed scans, taken about an hour apart, at the rate of 4 cm^{-1}/s. The line along the bottom is the difference in the two individual scans (offset slightly from zero)

more powerful than one might initially consider possible with a 16K minicomputer.

APPLICATIONS OF A DISPERSIVE SPECTROMETER–COMPUTER SYSTEM

Improving Sensitivity and Signal-to-Noise Ratio. Two simple operations that can be employed to enhance the signal-to-noise ratio of dispersive spectrophotometer data are spectrum-averaging and mathematical smoothing. Figure 7 illustrates the improvement obtained in a spectrum after mathematical smoothing, according to the procedure described by Savitsky and Golay (8, 9). In this procedure, a "moving" polynomial smooth is applied to the actual ordinate values, with a degree and span determined by the programmer. Our program utilizes a 21-point, fourth-degree polynomial. In this operation, the eleventh point of the spectrum is forced to conform to its twenty nearest neighbors by the least squares convention. The smoothed value then replaces the original datum, the 21-point interval is shifted one point to the right, and the next point is smoothed, and so on, thus the term "moving" smooth. The spectra in Figure 7 serve to illustrate the improvement in S/N obtained through the 21-point quartic smooth. The bottom plot ①, is the difference between the raw data ③ and the smoothed data ② for a fairly noisy, single-scan spectrum of an aqueous solution–germanium internal reflection prism system.

The process of applying a least squares smoothing function to noisy data is basically similar to the analog concept of an RC filter, except that the analog filter can only use data which have preceded the present measurement in time, and is thus one-sided. Since the spectrum is already completely stored in the case of mathematical smoothing, the process can use equal intervals of data both before and after the point being smoothed. Therefore, while mathematical smoothing may distort peaks which are narrower than the width employed in the computation, the distortion is one of peak height reduction rather than the distortion of the "right" obtained with RC analog filter networks. It is not necessary to smooth spectra prior to performing a baseline subtraction. This is valuable to the operator, in that he can leave his "raw" data intact on cassette files, and can smooth the difference spectrum after the subtraction operation is finished.

Figure 8 illustrates the value of spectrum averaging, showing internal reflection spectra obtained from 1 μg of p,p′-DDT on KRS-5. The system used included a 0.5-mm thick, 25-reflection KRS-5 prism, in a Harrick Scientific micro-internal reflection accessory. One microliter of 1000 ppm p,p′-DDT in hexane (Analabs) was deposited on one face of the small prism, and the solvent was allowed to evaporate.

The prism transmitted 18% of the normal energy at a fixed slit width of 0.7 mm and a spectral resolution of 1 cm^{-1}. Figure 8A shows the raw data obtained from a single, parallel polarized scan from 1150 to 1000 cm^{-1}, at 1× and 10× ordinate scale expansion, unsmoothed and smoothed. The smoothed spectrum shows how difficult it would be to correctly interpret such a high-noise spectrum. Smoothing is of little or no value where the S/N ≳ 1. The asterisks mark the locations of p,p′-DDT absorption bands in that region (1116 (weak), 1094 (strong) and 1016 cm^{-1} (strong)). The 1016 cm^{-1} band does not even show up in the 10×, smoothed spectrum. In Figure 8B, similar representations are shown for a computer-average of 25 scans. A S/N enhancement of $\sqrt{N}$ is expected for N averaged spectra, and an average of 25 scans should theoretically increase the S/N the same amount as one would obtain by slowing down the scan rate by a factor of five. This enhancement is quite evident in Figure 8B. The strong peaks are visible in the 10× spectra, both raw and smoothed, and in the smoothed, 10× spectrum some weaker bands are also apparent, including the one at 1116 cm^{-1}.

Scaled Spectrum Subtraction. One of the most difficult techniques to master in experimental infrared spectroscopy is differential spectroscopy. Differential spectroscopy involves the preparation of a reference cell system which matches the unwanted portion of the sample spectrum perfectly. For instance, aqueous solution spectra, in the transmission mode, require extremely thin (3 to 15 μm) cells of fairly high solute concentrations. Even with such thin cells, the water absorbs so much energy that a compensating cell must be placed in the reference beam, preferably one which can be adjusted until its transmittance matches that of the sample cell. Similar compensating technique are employed in many other applications in infrared spectroscopy, in order to observe small peaks in the presence of overwhelming solvent bands (10, 11).

Spectrum subtraction is an excellent alternative to differential spectrometry (12). Figure 9 illustrates spectrum subtraction in a way which provides some insight into the reproducibility of the spectrophotometer. The familiar spectrum shown in Figure 9 is actually two independent polystyrene spectra, taken about two hours apart, without any averaging or smoothing. The scans were run at 4 cm^{-1}/s, which is faster than the optimum speed for the 180's detector, with normal slit settings and automatic gain control (resolution about 8 cm^{-1} at 3000 cm^{-1}). The two superimposed polystyrene spectra were subtracted, and the difference is shown at the bottom of Figure 9. Differences exist at 1437, 754, 737, and 700 cm^{-1}, where the peak absorbances exceed 1.5. The noisier regions at 1200–1000 cm^{-1} and 600–500 cm^{-1} are due to relatively lower energy at the end of those grating ranges.
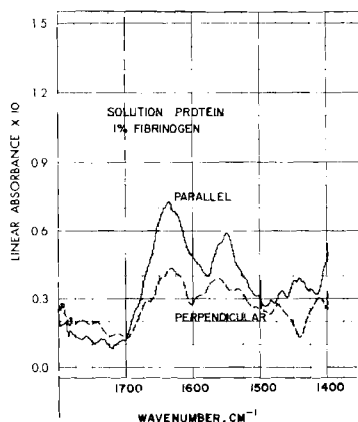
Figure 10. Parallel (TE) and perpendicular (TM) polarization difference spectra of fibrinogen in solution. Spectra obtained by subtracting spectra of citrate buffer solution

For years, infrared spectroscopists have relied on the positions and intensities of the N–H stretching, amide I (primarily C=O stretch), and amide II (C–N stretch + N–H) bands as aids in interpreting the secondary structures of synthetic polypeptides and natural proteins. These three absorption bands provide essentially all of the secondary structural information that can be obtained from the infrared spectrum of a polypeptide (13). Two of the bands, however, the N–H stretching band at approximately 3400 cm⁻¹ and the amide I band at about 1630–1650 cm⁻¹, are effectively obscured in aqueous solution spectra by the ~3380 cm⁻¹ and 1633 cm⁻¹ bands of liquid water. Various efforts to get around this problem have been employed, including the use of D_2O as a solvent, or employing a thin compensating reference cell, as discussed above. The thin compensating cell technique is inadequate for protein solution spectroscopy because of the concomitant requirement of high solute concentrations. D_2O solutions pose an additional problem, that of hydrogen-deuterium exchange. This results in a change in the intensity of the undeuterated amide II band, and formation of a deuterated amide II band at the same frequency as the HDO band (13).

Internal reflection spectroscopy, using a high refractive index, infrared-transparent prism such as germanium, provides the thin sampling region necessary to avoid the total loss of energy at the water peak maxima. For an internal reflection system, the "depth of penetration", d_p, is defined (14) as the distance into the solution where the evanescent field intensity decays to e^{-1} of its magnitude at the prism–solution interface. For germanium–water, at a 45° angle of incidence, this distance is 0.064 λ_0, where λ_0 is the *in vacuo* wavelength. Eighty-four percent of the peak intensity observed in an internal reflection spectrum is derived from absorbing molecules within one d_p of the prism–solution interface, and 95% of the observed peak intensity is due to molecules within two d_p. Thus for the amide I peak at 1630–1650 cm⁻¹, 95% of the band intensity comes from the 7750-Å thin region at the germanium–solution interface.

Using such a thin sampling region in combination with the signal-to-noise enhancing techniques discussed above, and by subtracting the solvent spectrum from the protein spectrum, the obscured amide I band of a protein can be separated from the overlying water band at 1633 cm⁻¹, as described below.

Figure 10 illustrates polarized internal reflection spectra of 1% fibrinogen in 0.17 M NaCl (0.34 M sodium citrate, 0.061 M sodium acetate, pH 7) in contact with a carbon optically transparent electrode surface at a controlled potential of −500 mV (to SCE). The protein solution was added to the cell by forcing out a citrate buffer solution. The spectra shown in Figure 10 were obtained by subtracting the polarized internal reflection spectra of the citrate buffer solution from the spectra of the 1% fibrinogen solution. The maximum transmission of the carbon OTE's is about 1.5% T at 1800 cm⁻¹, and the spectra used to compute the difference spectra in Figure 10 were averages over 25 scans, smoothed once. The 1633 cm⁻¹ band of water has an intensity of about 10 times that of the Amide I peak shown in Figure 10; however, the solution fibrinogen exhibits the expected Amide I and II bands at 1635 and 1550 cm⁻¹, respectively.

The ability to perform absorbance subtractions on data from conventional dispersive infrared instrumentation enhances the capabilities of such equipment tremendously. Many of the functions ascribed to Fourier transform infrared equipment, other than speed, really come down to operations performed by the *computer*, not by the interferometer.

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