Reprinted from THE REVIEW OF SCIENTIFIC INSTRUMENTS, Vol. 35, No. 9, 1108–1112, September, 1964
Printed in U. S. A.

Assembly and Performance of a Double-Beam Microscope Spectrophotometer from Commercial Instruments*

HEYMAN C. DUECKER AND ELLIS R. LIPPINCOTT
Department of Chemistry, University of Maryland, College Park, Maryland

(Received 27 March 1964; and in final form, 26 May 1964)

A microscope spectrophotometer assembled from commercially available instruments has been used to obtain visible and near-infrared spectra on selected specimen areas as small as 1 μ^2. A commercial spectrophotometer is coupled to a research microscope equipped with a photometer tube to which the photodetector of the spectrophotometer is attached. The miscroscope spectrophotometer can be assembled easily, quickly, and in a very compact form with a minimum of machine work. The performance characteristics are discussed, as well as some applications. The instrument was designed for spectral investigations of substances under pressure, but is equally well suited to the study of conventional microscope specimens. The incorporation of cameras and polarizing optics makes the instrument particularly suited to phase studies as well. Applications discussed are the shift of absorption bands with pressure, the determination of the pressure gradient in the diamond high pressure cell, and the determination of the spectra of microsections of stained biological specimens.

INTRODUCTION

AN instrument for the determination of the absorption spectra of selected microsections of biological specimens was first described by Caspersson[1] more than twenty-five years ago. The progress in instrumentation since then has recently been carefully reviewed by Wolken and Strother.[2] They have noted that the microscope spectrophotometers presently in use are laboratory-built instruments and are designed primarily for biological investigations.

The necessary components, viz. monochromator, light chopping device, microscope, photodetector, amplifier, and appropriate recording equipment can now be conveniently purchased. However, the assembly and matching of the components requires a considerable amount of machine work. The problems and delays inherent in such an assembly have made the construction of the microscope spectrophotometer feasible only in connection with a lengthy and comprehensive research program.

The present paper describes a recording double-beam microscope spectrophotometer which can be readily assembled from a commercial spectrophotometer and microscope which have an unusual physical and functional compatibility. The resulting instrument is very compact and is easily assembled. The instrument has the known performance characteristics of a proven spectrophotometer, and the microscope used offers a maximum of flexibility and precision including visual and photographic accessories.

Previously described instruments have been used for the investigation of biological specimens and, therefore, have conventional microscope requirements. The present instrument, however, has been designed for the investigation of substances in a diamond high pressure cell.[3] The physical dimensions of the high pressure cell impose the additional requirement of long working-distance optics. In general, a larger variety of optics for all spectral regions is available with conventional working distances, so that the instrument can be expected to perform as well, or better, in conventional microscopic investigations.

The instrument will be discussed on the basis of microscope spectrophotometry of substances in the diamond high pressure cell in the visible and near-infrared regions of the spectrum. Ultraviolet and conventional nonpressure microscopic applications will then be presented.

INSTRUMENTATION

After some investigation of commercial instruments, it was found that the Perkin-Elmer model 350 spectrophotometer[4] and the Leitz Ortholux microscope[5] were particularly suited to each other in several ways. In normal use, the light enters the Ortholux microscope horizontally and from the rear. This permits the mounting of the microscope in front of the sample compartment of the spectrophotometer so that the specimens can be conveniently mounted on the microscope. A photometer tube, designed for use with the Ortholux microscope in the Leitz single-beam spectrophotometer,[6] is available and simplifies the microsectioning operation as well as the coupling of the microscope to the photodetector described later.

The model 350 spectrophotometer has two matched photodetectors which permit the sample detector to be mounted on the photometer tube of the microscope. In

* Supported in part by grants from the U. S. Public Health Service, National Institutes of Health, and the U. S. Army Research Office, Durham.

[1] T. Caspersson, Skand. Arch. Physiol. **73**, Suppl. 8 (1936); J. Roy. Microscop. Soc. **60**, 8 (1940); Experientia **11**, 45 (1955).

[2] J. J. Wolken and G. K. Strother, Appl. Opt. **2**, 899 (1963).

[3] C. E. Weir, A. Van Valkenberg, and E. R. Lippincott in *Modern Very High Pressure Techniques*, edited by R. A. Wentorf (Butterworths Scientific Publications, Inc., Washington, D. C., 1962), p. 51.

[4] Perkin-Elmer Corporation, Norwalk, Connecticut.

[5] E. Leitz, Inc., 468 Park Avenue South, New York 16, New York, Bulletins 51-40a, 55-20.

[6] E. Leitz, Inc., 468 Park Avenue South, New York 16, New York, Catalog No. 52-D.1.

order to exactly match the two photodetectors, the model 350 is equipped with an I_0 compensator consisting of a bank of 18 trimmer potentiometers corresponding to different wavelengths throughout the spectral range. In the present application, the I_0 compensator is used to correct for the absorbance of the microscope optics. (When a particular diamond high pressure cell is used for an extended period of time, this procedure can be extended to correct for the absorbance of the diamond cell as well as the microscope optics.) An additional factor in the selection of the model 350 spectrophotometer is that it appears to be well suited to fluorescence microscope spectrophototometry. This modification is now being undertaken.

The assembled instrument is shown in Fig. 1 with some of the important visible components labeled. A schematic diagram given in Fig. 2 will aid in the discussion of the modifications and components.

A front surface mirror is placed in the sample compartment of the spectrophotometer so that the chopped monochromatic radiation, normally passing through the sample, is projected into the rear of the microscope and is reflected vertically into the condenser by the front surface mirror in the base of the microscope. A tube at the back of the microscope permits the mounting of a conventional microscope lamp so that the sample may be observed in white as well as monochromatic radiation.

The monochromator slit image is brought to focus at the sample, the size of the image being controlled by the selection of the condenser and the use of an optional monochromator beam lens (approximately 3.5×). The requirement of a long working distance is best satisfied by use of a coniscopic objective as a condenser. Objectives of this type may be obtained in magnifications between 5× and 40×, so that the monochromator slit image may be reduced in size by a factor of from 2 to 40 with a resulting (but not corresponding) increase in monochromator beam intensity. The size of the slit image at the sample is not critical as long as it is large compared with the area being scanned. Hence, the condenser system may be used primarily as a control of the monochromator beam intensity.

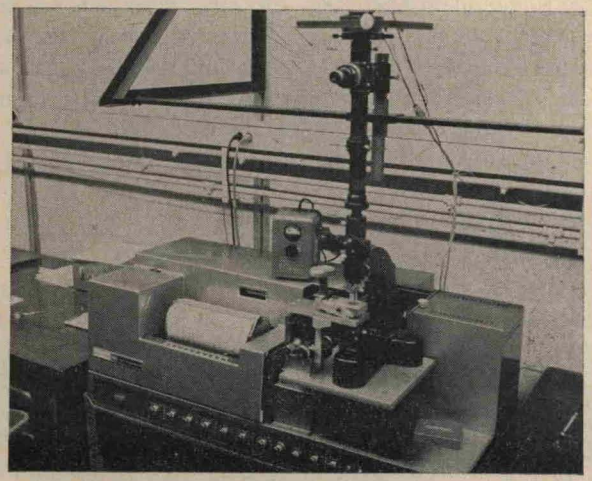

FIG. 1. Photograph of microscope spectrophotometer.

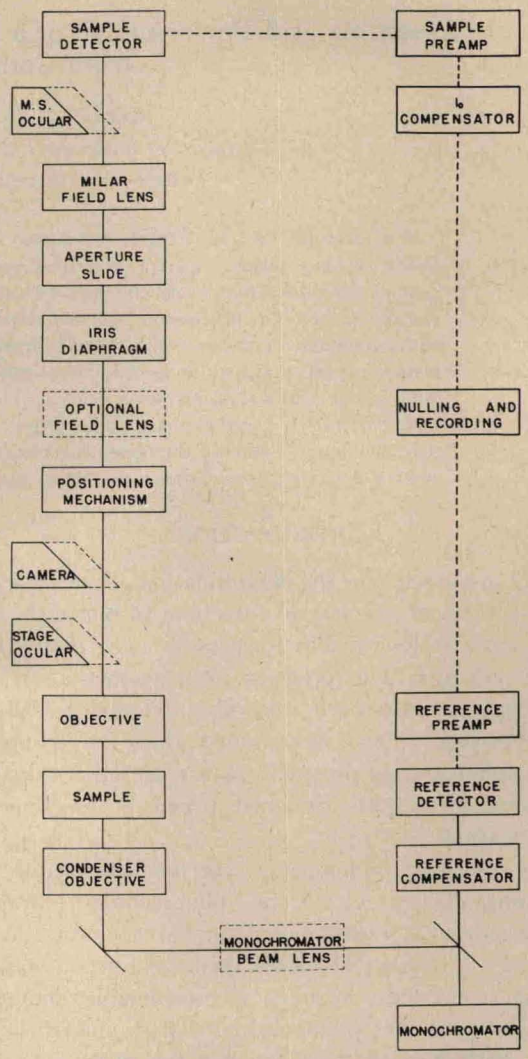

FIG. 2. Schematic diagram of microscope spectrophotometer.

The diamond high pressure cell may be mounted on a conventional microscope stage for observation at a given pressure. However, the force required to alter the pressure makes it desirable to mount the cell more firmly for investigations at different pressures. To satisfy this requirement, a more substantial stage is attached directly to the spectrophotometer.

The objectives used for observations in the high pressure cell are the same as those used in the condenser. The choice of the objective magnification is governed by the area of the specimen to be scanned and will be discussed in connection with the photometer tube.

The stage ocular, a binocular phototube with inclined 6× or 10× periplane eyepieces, is used in the preliminary

positioning of the sample and for positioning the monochromator slit image with respect to the sample. If petrographic or dichroic observations are to be made on a material, a polarizing binocular tube is used along with a polarizing condenser.

The camera may be used in the position indicated or may be attached to the microsectioning ocular to obtain a photograph of the area of the specimen selected for a spectral determination. Photographs of this type are particularly useful in the interpretation of spectra. Stage photographs of substances in the diamond high pressure cell are valuable to high pressure phase studies.[3] Using monochromatic radiation, such photographs have been used to determine pressure gradients in the high pressure cell.[7]

The photometer tube may be mounted on a base with the microscope or on a wall bracket as we have done (see Fig. 1). The bracket is made in such a way as to permit three-dimensional travel of the photometer tube. This provides for a simple alignment of the phototube unit, microscope, and monochromator.

A $\frac{1}{3}$ reducing field lens may be used to project the sample and slit image into the plane of the centerable iris diaphragm to help reduce the amount of stray radiation. If stray radiation is no serious problem, the reducing field lens may be removed and the image projected into the phototube or upper ocular after passing through the fixed aperture slide (containing aperture diameters of 1 through 5 mm) and a 6.5-cm Milar field lens. The area of the microsection selected is controlled by the size of the fixed aperture, the magnification of the objective, and the use of the reducing field lens. With the range of objectives described earlier, one can obtain microsections of almost any diameter between 10 μ and 3 mm.

The fixed aperture slide is also centerable, a feature which is generally used to control the position of the radiation striking the photodetector. Since all areas of the photocell do not give equal photoresponse, in order to compare spectral intensities, it is necessary to keep the size and position of the fixed aperture constant. In these cases the positioning of the sample image with respect to the fixed aperture is accomplished by means of the centering screws of the objective mount, or by a fine adjustment of the sample on the stage. The final selection of the microsection is observed through the microsectioning ocular and a photographic record of the section may be made at this point, as discussed earlier, if desired.

The standard photodetectors for the model 350 spectrophotometer are used, viz. EMI type 9529B end-on photomultiplier for the uv and visible regions and a lead sulfide cell for the near-infrared region. The detectors are taken from the spectrophotometer and mounted on the photometer tube after lengthening and properly shielding the leads. With the addition of a switch and another detector, it is possible to leave the photodetector in its original position. This permits the instrument to be used as a conventional spectrophotometer by rotating the sample compartment mirror. The reversion of the instrument to its original form is particularly helpful in making service checks and adjustments.

In the microspectrophotometry of specimens mounted in a cuvette or on a microscope slide, the coniscopic optics are replaced by achromatic or apochromatic objectives of greater magnification and with a greater resolving power. The coniscopic and achromatic objectives enable us to obtain spectra from 0.36 to 2.3 μ.

Presently, the range of the instrument is being extended into the ultraviolet region by the use of Bausch & Lomb "Grey-Polaroid" 20× long working-distance reflection objectives in place of the coniscopic objective and condenser. After substitution of a fused silica upper field lens, it should be possible to get spectral determinations from 0.2 to 2.7 μ. Reflection objectives of higher magnification (up to 300×) are now available for conventional microscope investigations throughout the same spectral range.

PERFORMANCE

Since many of the performance characteristics (such as stability, photometric accuracy, and photometric reproducibility) are virtually the same as they are for the model 350 spectrophotometer, only those which are grossly altered will be discussed here.

A demonstration of the use of the I_0 compensator is given in Fig. 3. The absorption due to the optical system and a typical diamond cell is given along with the adjustment of the I_0 line to correct for these. The further expansion of the usable range of the instrument by use of reflection objectives has been mentioned earlier.

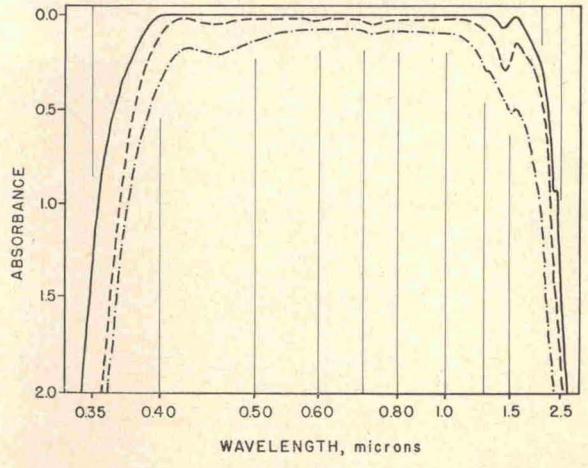

FIG. 3. Typical I_0 compensation curves; – – –, spectrum of optical system; – · –, spectrum of diamond cell in optical system; ——, I_0 correction. Absorbance is defined as log I_0/I.

[7] E. R. Lippincott and H. C. Duecker, Science 144, 1119 (1964).

The effectiveness of the magnification of the monochromator beam intensity mentioned earlier is partially reduced by the absorption and reflection losses within the optics. Thus, the total amount of light striking the photocell is less than when the spectrophotometer is used in a conventional manner. However, since we are able to project the energy onto a more sensitive part of the photocell, we get an increase in photoresponse. Photographic comparison of the light intensity at the photocell ports indicates an effective increase in spectral response of about 20%. However, this is affected considerably by the alignment of the instrument and the photocell, so that one might generally be concerned with a loss rather than a gain in photoresponse if the proper care is not taken. It appears that the same range of optical densities can be covered as with the conventional spectrophotometer.

The effect of the size of the area selected on the resolution of the spectrum is almost negligible for areas of 10 μ^2 or greater. There is some loss of resolution however, if the area is reduced to 1 μ^2. Figure 4 gives a comparison of spectra taken of the holmium oxide glass wavelength standard using scan areas of 1 and 10 μ^2 as indicated. Otherwise, the resolution appears to be about the same as that of the model 350 spectrophotometer alone, viz. 1.0 mμ or less in the visible region.

APPLICATIONS

The instrument has been used in the investigation of the effects of pressure on the spectra of a number of substances. Some materials, e.g., nickel dimethylglyoxime, have an absorption band in the visible region which shifts with pressure to a longer wavelength (see Fig. 5). Another group of materials, such as the thallous halides, has an extremely strong absorption band in the ultraviolet which shifts into and through the visible region as pressure is applied. Some substances have sharp phase transitions, e.g. mercuric iodide, with perhaps some other spectral

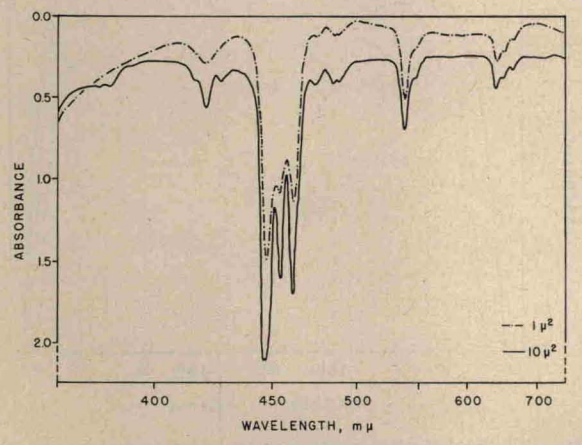

FIG. 4. Effect of scan area on resolution using areas indicated. Lower curve is shifted to make a comparison possible.

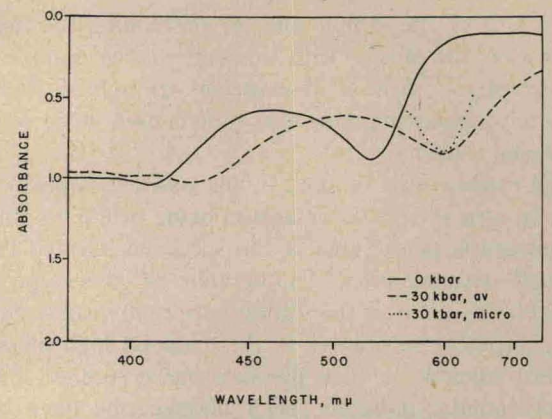

FIG. 5. Spectra of nickel dimethylglyoxime in diamond cell; ——, entire sample at 0 kbar; – – –, entire sample at 30 kbar; · · ·, 10-μ-diam microsection at 30 kbar.

effect superimposed. In Fig. 6 the absorption edge of the red and yellow (high pressure) form of mercuric iodide is shown along with a subsequent shift of the edge with pressure. An absorption spectra of the entire sample is a mixture of the spectra of the red and yellow forms and the component spectra cannot be determined without the use of the microscope spectrophotometer. Figure 6 gives the spectra for the red and yellow components at an applied pressure of 12 kbar, as well as for the brownish region beginning to form in the highest pressure zone.

The two other types of spectra may be complicated in a similar manner. However, for the first type, i.e., with nickel dimethylglyoxime, the average spectrum (taken from the entire sample) is apparently not too objectionable, since

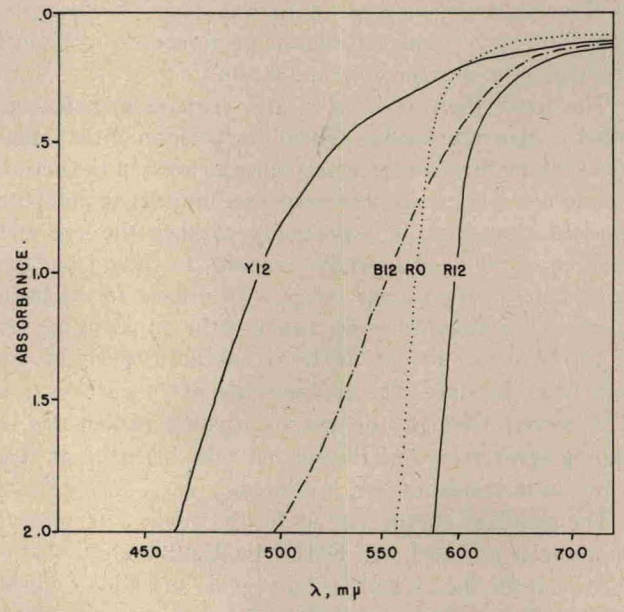

FIG. 6. RO spectra from red mercuric iodide at 0 kbar. Spectra of different areas of mercuric iodide in diamond cell at applied pressure of 12 kbar as follows: R12 from red region near phase boundary; Y12 from yellow region near phase boundary; B12 from brownish high pressure region forming at the center of the sample.

the location and shift of the absorbance peak as a function of pressure are the same as those reported by Zahner and Drickamer[8] for the same material under a presumably uniform pressure. Nevertheless, the absorption bands are considerably sharper and narrower when measured in a microscope spectrophotometer as shown by the dotted line in Fig. 5.

Now since the position of the absorption band is known as a function of pressure, the microscope spectrophotometer has been used to determine accurately, for the first time, the pressure gradient of a material such as nickel dimethylglyoxime in the diamond high pressure cell.[7] Using the instrument described, spectral measurements corresponding to as many as ten thousand microsections of the sample in the pressure cell can be determined. In practice, it was found that about seventy measurements gave enough information to draw pressure contours maps of the diamond surface (using the position of the absorbance peak vs pressure relation). An extension of the pressure gradient studies into materials of different compressibilities indicates that determinations of the compressibilities of a material as a function of pressure can probably be made using this technique.

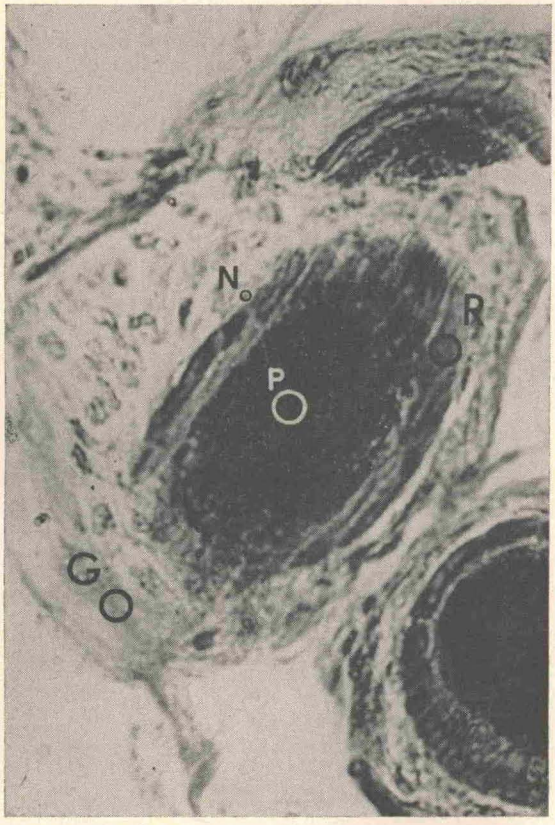

FIG. 7. Photograph of stained thin section of sheep hair follicle.

[8] J. C. Zahner and H. G. Drickamer, J. Chem. Phys. 33, 1625 (1960).

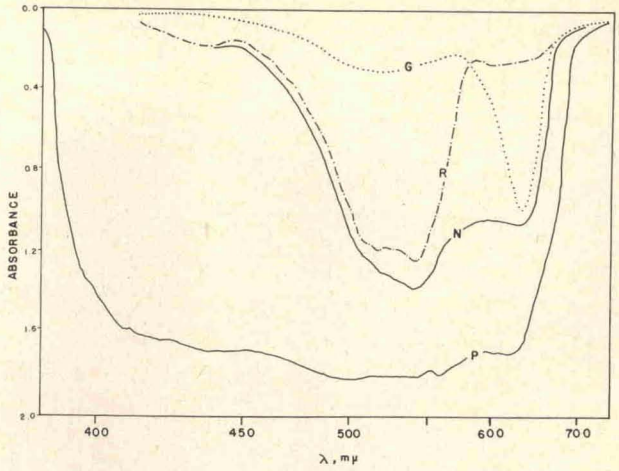

FIG. 8. Spectra of selected regions indicated in Fig. 7 from sheep hair follicles.

The application of the microscope spectrophotometer to conventional microscope specimens may be demonstrated with a stained thin section taken from the hair follicle of a sheep (see Fig. 7). The sample is stained with safranin and fast green dyes, but since the reproduction is not in color here, the color of the areas is appropriately designated. The green area (G) is connective tissue, the sebaceous cells (R) surrounding the hair follicle are red, the follicle tissue (P) is a deep purple color, while the nuclei of the tissue cells (N) are reddish purple. The areas designated were selected for the spectral determinations given in Fig. 8. The scan area for all measurements was 10 μ^2 except an area of 1 μ^2 is used for the determination of spectrum of the nuclei. These spectra were taken using a 100× achromatic objective.

Other applications of microscope spectrophotometry discussed by Caspersson[1] and Wolken and Strother[2] include studies in pigment chemistry, photoreceptors in living cells, and the metabolism of living cells. The use of microanalytical spectroscopic techniques has been discussed by them as well as by Mason.[9] It is expected that the present instrument equipped with the proper optics may also be satisfactorily used for these applications.

In general, we can say that the microscope spectrophotometer is an aid to spectroscopic analytical problems where very small quantities of material (as small at 10^{-9} g) are available or where the sample is nonhomogeneous. In addition, the instrument described herein can be used for ultraviolet, visible, and near-infrared spectral determinations on samples which require a working distance of up to 15 mm. In this respect, the instrument is adaptable to spectral observations of materials under pressure as well as in a vacuum, and at very low as well as at elevated temperatures.

[9] W. B. Mason in *Conference on Submicrogram Experimentation, Arlington, Virginia, 1960*, edited by N. D. Cheronis (Interscience Publishers, New York, 1960), pp. 293–310.