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CORDIS CORPORATION
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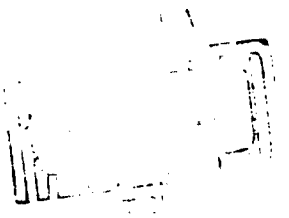
Miami 37, Florida



Detection and Measurement of Antigen-Antibody Reactions

ANNUAL PROGRESS REPORT

Period Covered:
1 October 1961 - 30 September 1962

Principal Investigator:
William P. Murphy, Jr., M.D.

Institution:
Cordis Corporation

Detection and Measurement of Antigen-Antibody Reactions

OTSG Contract No.
DA-49-193-MD-2203

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Cordis Corporation
241 N. E. 36th Street
Miami 37, Florida

30 September 1962

ABSTRACT

1. Preparing Institution:

   Cordis Corporation

2. Title of Report:

   Detection and Measurement of Antigen-Antibody Reactions

3. Principal Investigator:

   William P. Murphy, Jr., M.D.

4. Number of pages: 32
   Number of illustrations: 4
   Date: 30 September 1962

5. Contract Number:

   DA-49-193-MD-2203

6. Supported by:

   U. S. Army Medical Research and Development Command
   Department of the Army
   Washington 25, D. C.

7. Descriptive abstract:

There is included in this report a description of work performed under subject contract during the period October 1, 1961 - September 30, 1962. Pertinent material from the Progress Report, dated June 9, 1962, has been repeated to give a complete presentation. Those who have read the Progress Report will find new material in Sections II-C, D E and III.

Abstract, cont.

The following accomplishments have been made during the year:

1. A technique has been developed for growing turnip yellow mosaic virus and producing antibody in rabbits, providing a suitable immunological system for the current investigation.

2. Successful experiments have been performed using labelled diphtheria toxoid as a reagent to detect diphtheria antibodies.

3. Disintegration of bacteria to form fragments of low molecular weight suitable for the technique under consideration has been achieved and antibodies to the cell walls prepared.

4. Instrumentation sensitivity and reliability have been improved by changes in the optical and detecting systems, as well as by the incorporation of additional controls.

There is also described the program of work to be accomplished in the period October 1 - December 31, 1962.

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I. INTRODUCTION

The current investigation concerns application of a technique developed by Walter B. Dandliker, Ph.D., Professor of Biochemistry, University of Miami School of Medicine.

This involves measurements of fluorescence polarization and/or intensity measurements made on solutions which contain fluorescent-labelled antigens or antibodies. The antigen is labelled if it is desired to detect an antibody; conversely, the antibody is labelled if the detection of an antigen is required.

This technique was studied in great detail by Doctor Dandliker using fluorescein-labelled ovalbumin and rabbit antifluorescein ovalbumin. Fluorescence polarization measurements were made with a modified light-scattering apparatus. His need for more sophisticated and accurate optical equipment soon became obvious and an improved polarometer* was built for him by Cordis Corporation.

The success of Doctor Dandliker's

*Funded under NIH Grant RC-8970.

results led to speculation about a wide variety of applications of the basic technique. These varied from specification of allergenic reactions to detection and identification of microorganisms. These possibilities aroused the interest of the Office of the Surgeon General and resulted in the current contractual effort with Doctor Dandliker as consultant and Cordis Corporation as contractor because of the strong emphasis to be placed necessarily on instrumentation and application engineering.

Initiation of work under the contract was authorized October 1, 1961. The microbiological facility at Cordis, essential to the efficient prosecution of the contract was improved during the period October 1 - December 31, 1961, and additional personnel recruited. At the same time, work was carried out on improvement of the instrumentation system which had been developed and constructed in prototype form.

Early in January, 1962, the program of measurements to be outlined in Section II of this

report was initiated. Following this description is an outline of work to be performed during the next quarter, October 1 - December 31, 1962, using funds made available through an extension of the present contract.

The positive results obtained to date indicate the desirability of continuing the current research effort and a proposal for follow-on work has been made.

II. TECHNICAL PROGRESS

A. Relative Lifetime Estimates and Absorption and Emission Characteristics of Selected Fluorescent Dyes.

The choice of fluorescent label for a particular application of fluorescence polarization depends upon the range of molecular sizes involved in the antigen-antibody reaction and also upon the intensity and wave length regions of the background fluorescence due to adventitious impurities.

Measurements have been made on two labels, fluorescein and rhodamine. While the fluorescent intensity of rhodamine is lower than that of fluorescein, its emission characteristics offer the advantage of low background radiation. The pertinent absorption and emission curves are shown in Figs. 1 and 2.

The fluorescence polarizations for fluorescein-ovalbumin and rhodamine-ovalbumin are 0.141 and 0.142, respectively, not significantly different. The values of the polarization for free fluorescein and rhodamine extrapolated to infinite

FIGURE NO. 1

ABSORPTION AND EMISSION OF FLUORESCEIN

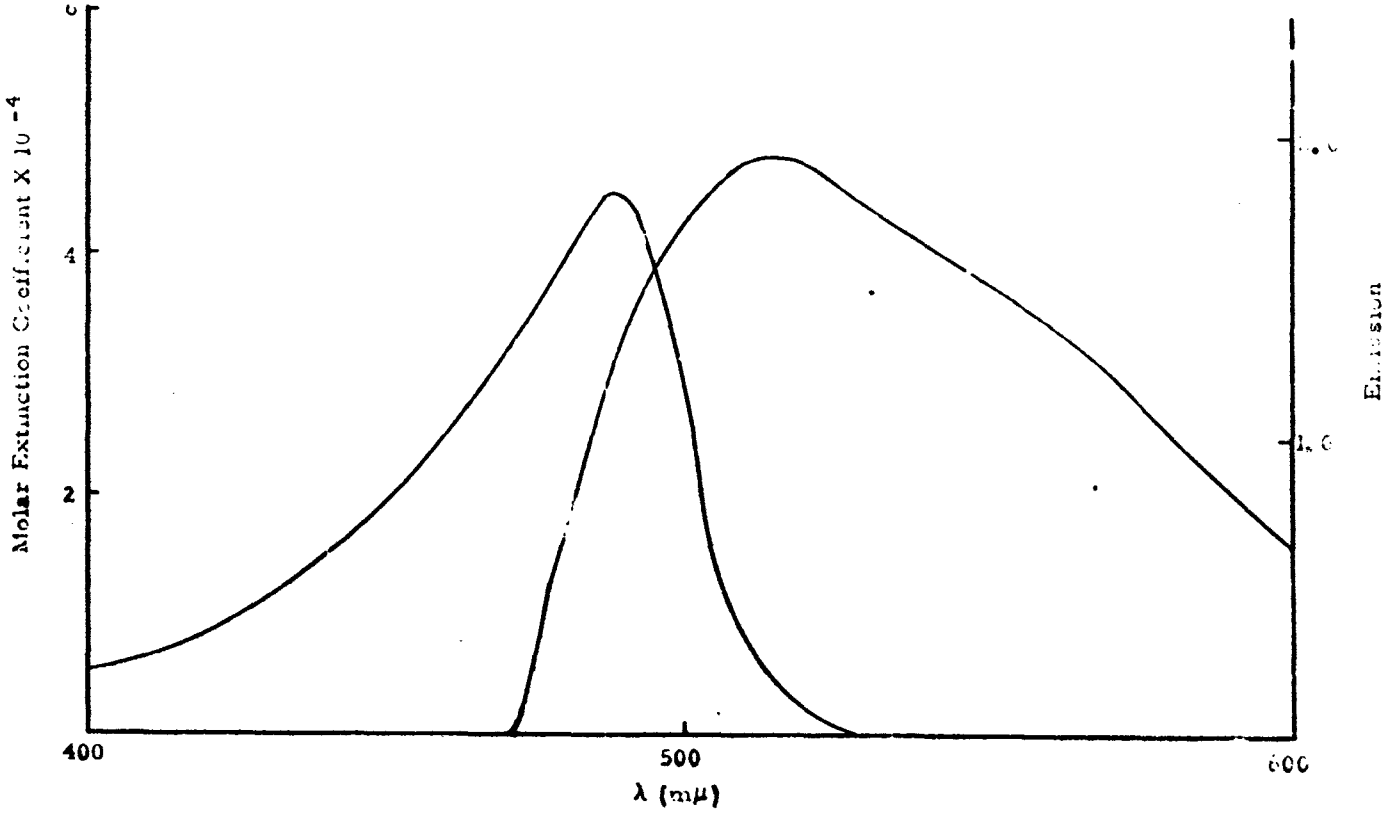
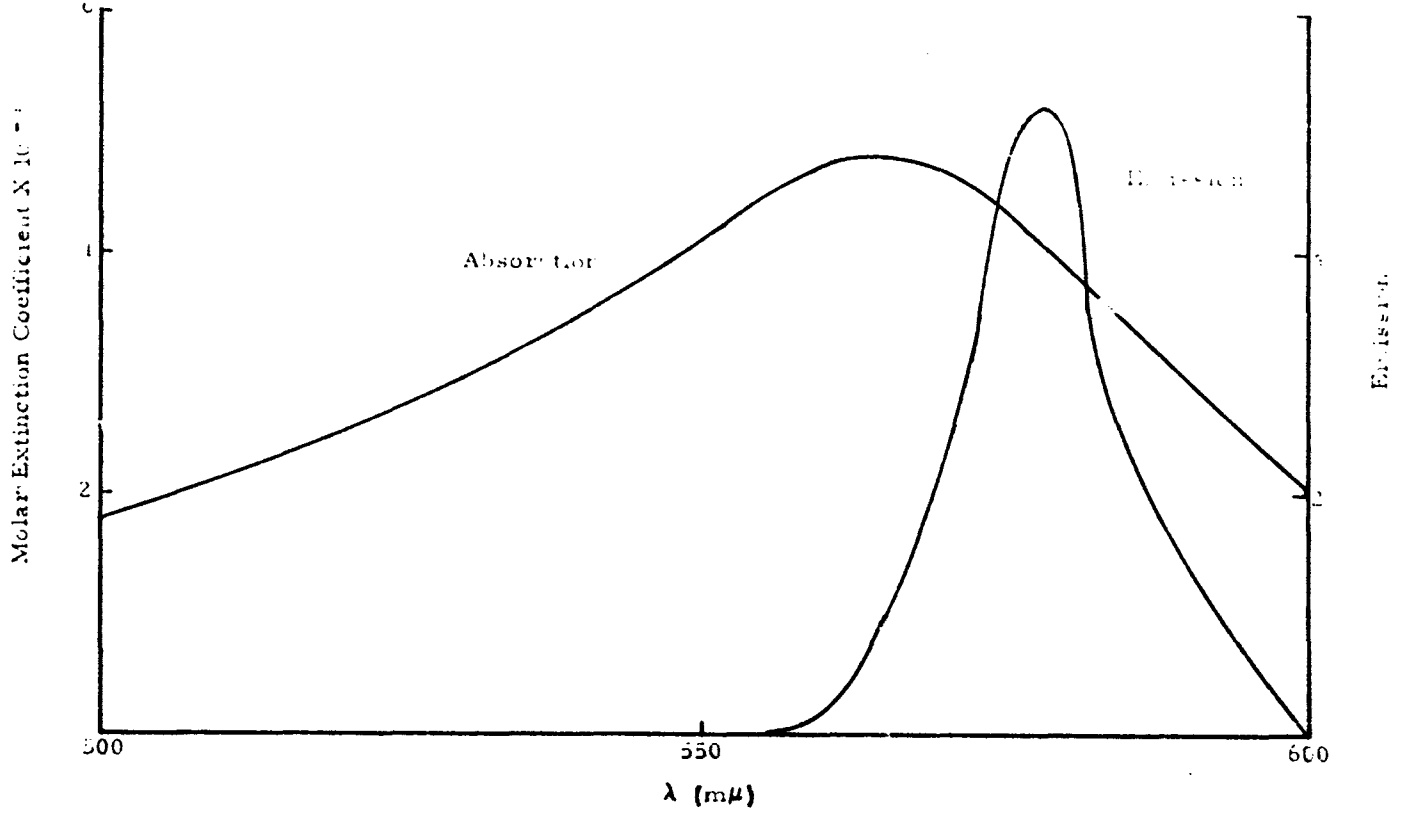


FIGURE NO. 2

ABSORPTION AND EMISSION OF CHLOROPHYLL



viscosity (in glycerol) are 0.231 and 0.193 respectively. Tentative interpretation of these data by the theory of Perrin for spherical molecules indicates that the excited lifetime of fluorescein is about 1.5 times that of rhodamine. However, the lower anisotropy of fluorescein appears to offset the longer lifetime and make the two dyes suitable for the same ranges of molecular weight.

B. Detection of Virus and Antiviral Antibodies

The immunological system of this investigation consisted of turnip-yellow mosaic virus and rabbit antibody. Approximately one gram of the virus was produced by growing it under laboratory conditions in Chinese cabbage, followed by isolation and purification in the ultracentrifuge by the method of Matthews (1), as modified by Hirth et al (2). Consideration has also been given to use of the nucleic-acid-free protein component of turnip-yellow mosaic virus studied by Kaper and Steere (3) and by Kaper (4). Methods for the dissociation of this virus follow closely that of Frankel-Conrat (5) which utilized 67% acetic acid for breaking down tobacco-mosaic virus. Recently Harris and Hindley (6) have adapted this procedure to turnip-yellow mosaic virus to obtain subunits of molecular weight 21,300. These fragments should be of an ideal size range for fluorescence polarization applications.

Antibody to turnip-yellow mosaic virus was made in rabbits by a series of eight injections, each consisting of 5 mg of the virus administered on alternate days. After waiting a week, the rabbits

were bled but very little antibody was found. After a rest period of two months, two booster shots of 5 mg each, given on alternate days and followed by a week's wait, resulted in antisera with a very satisfactory content of precipitating antibody. The sera showed visible precipitation with virus even when diluted one hundred fold. When used undiluted, one milliliter of serum yielded an antigen-antibody precipitate containing about 0.5 mg of nitrogen. Labelling of the rabbit antibody with rhodamine gave a product still highly reactive to the virus.

C. Diphtheria Toxoid and Antitoxin

In the following described experiments, the goal was to adapt the fluorescence polarization technique to the detection and measurement of antibodies to diphtheria toxoid or toxin.

Detailed investigation of the methods and conditions for labelling the diphtheria toxoid with fluorescent labels, especially with rhodamine, has brought to light the information needed to obtain stable labelling and, at the same time, to effect a further purification of the toxoid. The reagent finally obtained is a dry, comparatively stable powder which can be redissolved when needed and which can be applied, using the fluorescence polarization method, to the detection and measurement of diphtheria antitoxin in concentrations of as little as one to five units.

Rhodamine-B-isothiocynate was introduced and formed a labelled toxoid with sufficient fluorescence to permit high sensitivity of detection and measurement. Under normal conditions, this labelled material demonstrated a polarization which was a function of time. Several alternatives presented

themselves as possible explanations for the unstable polarization observed:

(a) surface denaturation of the toxoid;

(b) dissociation of the toxoid at very low concentration;

(c) an instability of the label on the toxoid resulting in partial release into a free state; and

(d) the presence of colloidal micelles of unreacted dye which would slowly redissolve when the concentration was lowered.

It has been established that (d) is the correct explanation. Apparently rhodamine, like certain other dyes, has a pronounced tendency to form colloidal particles of about the correct size to pass easily through the Sephadex column used. By migrating with the protein, they make it appear that the latter is heavily labelled. When such preparations are highly diluted, the anomalous, slow drop in polarization is observed. This colloidally dispersed dye can now be removed by a succession of filtrations, first on a Millipore type HA filter and then on a Corning very fine and ultra fine sintered glass filter. There is indication that a series of graded filters of the Millipore type would serve the same function, large amounts

of material being left on such a filter with 450 mμ pore size, a lesser amount on a 100 mμ one, almost none on a 10 mμ filter.

The diphtheria toxoid, once labelled and purified in the above manner, can be run through a Sephadex column to remove electrolytes and then freeze-dried to a stable pink-colored powder. This material redissolves very rapidly in neutral, buffered saline and reacts with diphtheria antitoxin in the flocculation test.

Most of the work in this research has been carried out with diphtheria toxoid lot PT119P* which has a concentration of about 1970 L_f per ml and which contains about 1.63 mg of nitrogen per ml. This toxoid then, on a nitrogen basis, seems to be about 41% pure when compared with the best preparations previously obtained (7). A considerable amount of dark-brown pigment is present in this preparation. This, fortunately, is removed during a

*Obtained through the courtesy of the Antitoxin and Vaccine Laboratory of the Department of Public Health of the Commonwealth of Massachusetts.

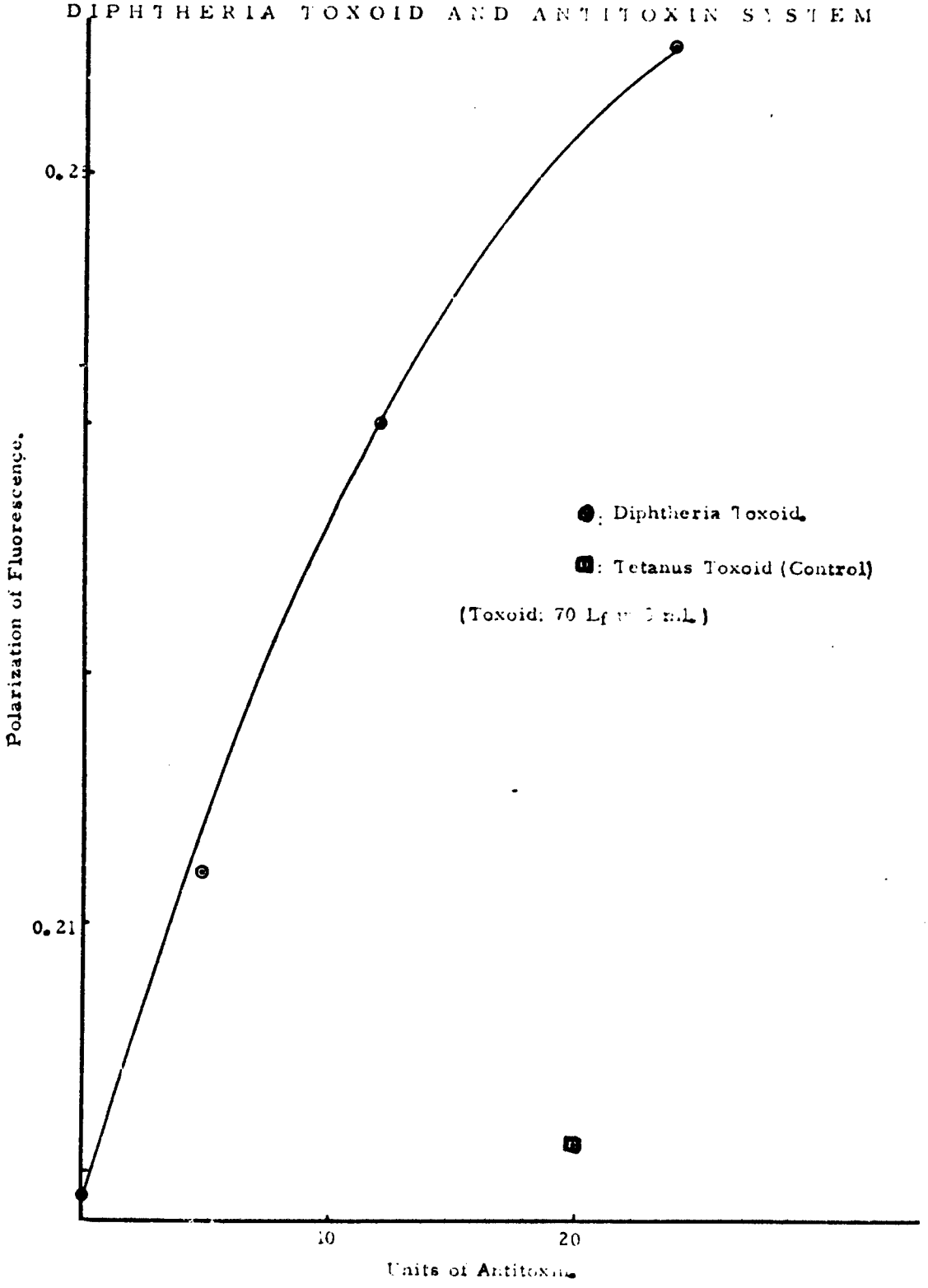
treatment with Sephadex following the labelling procedure and represents an additional step of purification. The final material, which is obtained in a dry state after labelling, was found to be about 1.5 times as active, on a dry weight basis, as the initial material and is probably about 60% pure. This stable, rhodamine-labelled toxoid (Rh toxoid) has been used to detect and measure the presence of antibodies to diphtheria.*

Fig. 3 indicates the type of data obtained. For this purpose, an experiment was set up with varying quantities of antitoxin in the cuvette to which was then added a constant amount of toxoid yielding approximately 20 L_f per ml (total volume 3 ml). The range of antitoxin varied from zero to 24 units. The polarization can be seen to be a smooth function of the number of units of antitoxin and, with the precision presently obtainable, it should be possible to measure 5 units of antitoxin with a precision of plus-or-minus one unit, larger quantities with even better accuracy. At the

*The antibody preparation was antitoxin obtained through the courtesy of the New York State Department of Health.

FIGURE NO. 3

DIPHTHERIA TOXOID AND ANTITOXIN SYSTEM



current stage of development, about one unit of antitoxin appears to be detectable. Higher sensitivity can be expected in future investigations by decreasing stray-light levels in the instrument and by using more highly purified toxoid preparations.

A standard flocculation test between diphtheria toxoid and antitoxin would require perhaps 20 units of antitoxin in a single tube (7a), although the quantity could be lowered drastically by substituting micro tubes. Investigation of the same system, using the quantitative preciptin test, utilizes about 400 L_f units of toxoid and 300 units of antitoxin for maximum precipitation. From 300 units of antitoxin, approximately 0.48 mg of antitoxin nitrogen is obtained in the precipitate, indicating that one unit of antitoxin contains about 1.6 micrograms of nitrogen. These data were taken from Kabat and Mayer (7b). A recent study of the diphtheria system, using the quantitative preciptin test and the micro-Kjeldahl method, gave useful data in the region of the equivalence point with quantities of toxin of the order of 500 units (7c).

The specificity of the reaction between Rh toxoid and diphtheria antitoxin may also be seen

from Fig. 3, which shows one plotted point, representing 20 units of tetanus antitoxin, giving a polarization very close to that of the blank. It is possible that there is a very weak cross reaction between the tetanus and diphtheria systems but further investigations would be needed to verify this.

In order to be able to compare results obtained with horse diphtheria antibody and rabbit antibody, the latter has been prepared. Commercial alum adsorbed toxoid from Lilly Laboratories is being used for immunization. A first series of about 16 injections, followed by bleeding 5 to 7 days after the final injection, gave a titer of about 4 units of antitoxin per ml and, after the second course of injections, about 36 units. Work using these sera will be undertaken after the horse system is more thoroughly understood.

D. Preparation of Reagents for Fluorescence Polarization Measurements from Bacteria

While most parts of bacteria are believed to contain antigenic materials, the cell walls and flagella, when present, probably offer the best opportunity for obtaining preparations free from interior cytoplastic constituents. Based upon the careful work of Weibull (8), methods are available for the isolation of flagella from certain bacteria; for example, Proteus vulgaris. Purified flagella contain a high proportion of lysine and thus should be readily labelled by isothiocyanates (9).

Weibull also describes the splitting of flagella to form a relatively low molecular-weight material, flagellin. The latter, with a molecular weight of about 40,000, is of the proper range and size for fluorescence polarization work. Its purification has been described by Koffler and Kobayashi (10).

In attempting to utilize flagellin in this way, we have followed the methods of flagella isolation used by Weibull. However, the volume of culture media needed to obtain sufficient flagellar material for antigen-antibody studies is, at present, prohibitive.

We have, therefore, centered our attention upon antigens in the cell walls, more readily prepared in sizable quantities.

A variety of equipment and procedures are at present available for the rupture of bacterial cells, preliminary to separation of cell wall material from cytoplastic content. Of these apparatuses and procedures, utilizing two organisms, Bacillus megaterium and Micrococcus lysodeikticus, we have tested the Branson Sonifier, a freezing and thawing technique, shaking with ballotini in an ordinary mechanical shaker, extrusion through an orifice at 30,000 psi, ultrasonic apparatus operating at 1,000 megacycles and the Mickle disintegrator. The most satisfactory procedure involved use of the Mickle disintegrator.

For effective disintegration, the bacteria must be washed in water and disintegrated while still suspended in water. When salts are present, there seems to be exerted on the bacteria a remarkable protective action, probably due to osmotic forces.

The disintegration has been followed

by turbidity measurements. These reveal a rapid decrease in turbidity, approaching a constant value. When no further changes take place, microscopic examination of stained preparations confirms complete destruction of the bacterial bodies.

After disruption, the cell-wall material must be freed of cytoplasmic constituents. This purification has been the subject of considerable study, especially by Salton and others (11), (12). The cell-wall material usually was washed by centrifugation, either in dilute phosphate buffers or in one molar sodium chloride, and then in distilled water until the chloride ion disappeared. This is substantially the procedure we have followed for B. megaterium and M. lysodeikticus.

Once the cell-wall fragments are obtained, these can then be degraded to relatively low molecular weight fragments, expected to be suitable for our purpose by either lysozyme or proteolytic enzyme action. Most of the results obtained concern the action of lysozyme. Both B. megaterium and M. lysodeikticus have been found in the past to be

very susceptible to lysozyme action, but the strain of B. megaterium which we first used was found to be resistant to this enzyme. As a result, work was concentrated on M. lysodeikticus which is extremely susceptible to lysozyme action, the cell-wall fragments being degraded very quickly in phosphate buffer, pH 7 (0.1 M).

Successful production of antisera to cell walls of M. lysodeikticus has been achieved. The cell walls appear to be rather weakly antigenic and, after a series of 11 injections lasting over a month, only the extremely weak, perhaps questionably agglutinating antibody could be found. However, even after as little as six injections, complement fixation tests* showed that the titer of antibody to cell-walls was approximately 20 times normal. These results are encouraging and seem to indicate either that the type of antibody being manufactured does not lead to agglutination or that the amount is still very small and a longer course of injections is needed. Routine complement fixation test procedures, to follow the antibody titer, are being set up in the Cordis laboratory.

*These tests were carried out by Robert A. Nelson, Jr., M.D., Senior Investigator, Howard Hughes Medical Institute, Miami.

In order to retain sensitivity to lysozyme in the Micrococcus strain, it has been necessary to reseed the experimental cultures frequently with lyophilized organisms and to use the growth media recommended by Litwack and Pramer (13). The best condition for disintegration in the Mickle Disintegrator is found to be 3.5 ml of a 10% cell suspension in distilled water and an equal volume of glass beads No. 10 or 12. Twenty to thirty minutes shaking gives 95 to 98% cell disruption. The degree of disruption has been checked by microscopic examination of Gram-stained smears.

Further work on the degradation of cell walls by lysozyme has been carried out. Digestion of cell walls of M. lysodeikticus was made with about 10 gammas of lysozyme per ml for about 48 hours, after which time little further change occurred. This material was then incubated with about 2×10^{-4} molar sodium lauryl sulfate (14) which quantitatively inactivates the lysozyme. The sodium lauryl sulfate can then be removed by dialysis leaving a product of degraded cell walls probably contaminated with irreversibly inactivated, denatured lysozyme.

In addition, it has been found that papain activated with cysteine also results in cell wall degradation as judged by optical density changes. The action of a proteolytic enzyme like papain should result in material which is largely carbohydrate in nature. Lysozyme digestion should give one which perhaps is largely peptide. A comparison of activity of these two types of material in agglutination, precipitation or complement fixation, should indicate where the chief antigenic sites are located, thereby providing appropriate materials for the fluorescence technique.

E. Improvement of Equipment

The fluorescence polarometer which had been developed for Doctor Dandliker for more effective fluorescence polarization measurements is illustrrted in Fig. 4. The functions of the various components of this instrument were as follows:

> Light from a mercury arc source is focused to a small spot within the solution cell. A suitable filter, either of the colored glass or interference type, isolates the desired portion of the spectrum. The polarizer immediately before the cell is optional since measurements may be made with either linearly polarized or unpolarized light. The fluorescent light from the solution is viewed in a direction at 90° to that of the incident beam. Scattered or parasitic light of the incident wave length is removed by a second filter, suitably chosen to transmit the fluorescent light. A second lens collects the light and focuses it through the Cotton prism, onto the chopper. The function of the Cotton prism is to separate the vertically-polarized and horizontally-polarized components of the fluorescent light so that they are transmitted alternately by the chopper. This results in a series of electrical pulses from the phototube which are then amplified and compared electrically in the detector.
>
> From the ratio of the horizontal to the vertical component, the polarization is readily computed. The addition of one manually-rotated polarizer, with angular scale as shown, makes it possible to utilize the photocell as a null detector. This follows because, for one position of the polarizer, the two pulses seen by the photocell are of equal magnitude. The ratio of the two

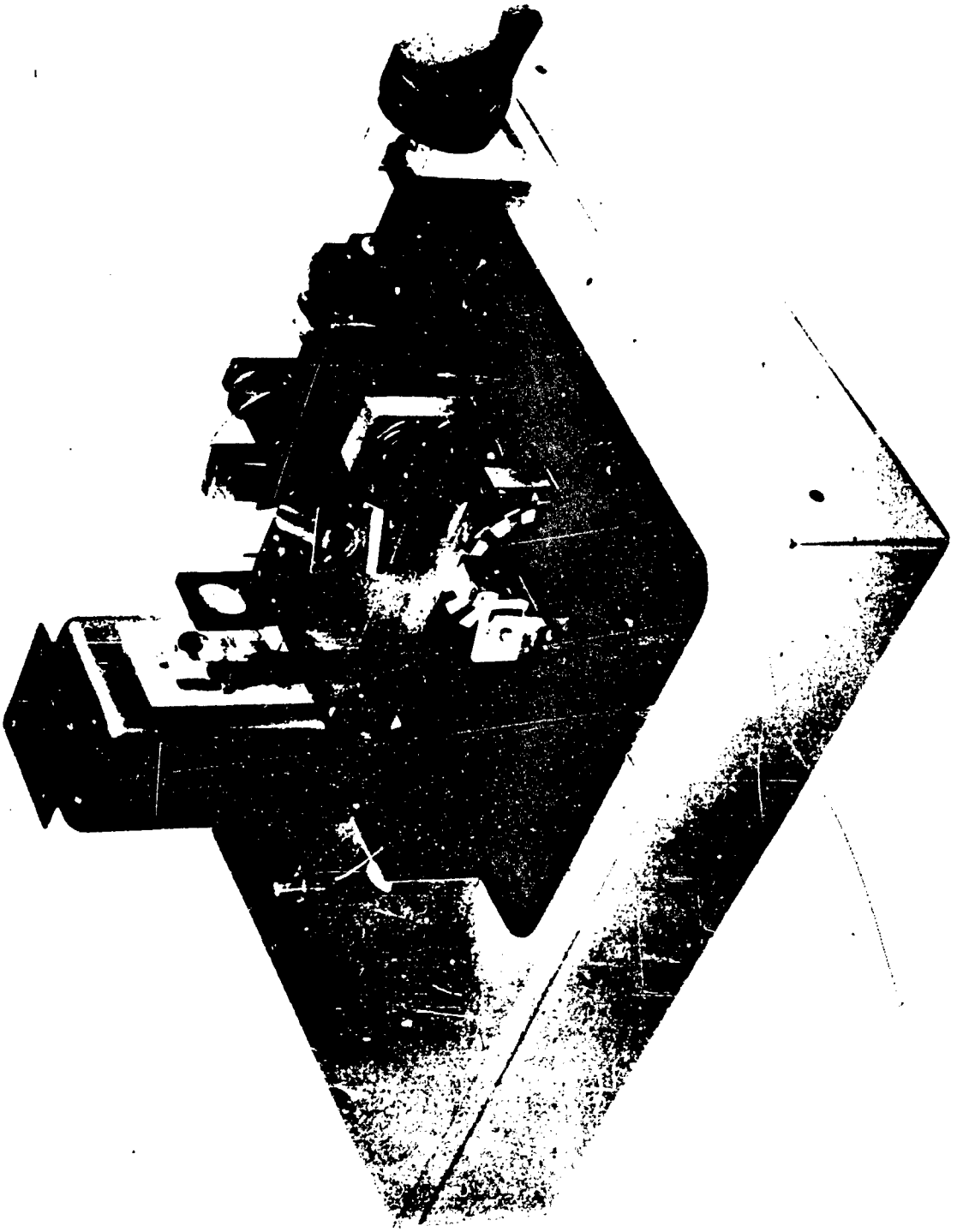


Fig. 4 - Fluorescence Polarometer

components emergent from the Cotton prism can be computed by the cosine-squared-law after reading the position of the manually-rotated polarizer.

A number of improvements and changes have been effected in the course of the current contractual effort. One important difference involved the substitution of a Rochon prism for the Cotton prism of the "analyzer". Since the ordinary beam of the Rochon prism is undeviated, this design provided a more convenient means of switching the light entering the chopper and photodetector from a vertical to a horizontal polarization. In this way, the unwanted polarization is discarded and the selected one compared to an unpolarized reference beam. This latter is obtained from a polished sphere illuminated by the entrance beam. The intensity level of the reference beam is approximately matched to that of the fluorescence by placing a fixed aperture mask over the objective lens. Fine measurement is then accomplished by rotation of a circular, neutral-density filter, the angular position of which can be measured to one part in 1,000. No polarizer is used in the entrance beam for reasons mentioned above.

The instrument was originally constructed with a lead sulphide detector. This was mounted in a special cone-channel optical system directly illuminated by the main beam. At the same time, the reference beam was introduced through the back of the lead sulphide cell in phase opposition. This system was adequate for high levels of intensity. But sensitivity, for low levels and at the wave lengths of principal fluorescence of fluorescein, was found to be too low. The lead sulphide cell has, consequently, been replaced by a photomultiplier tube of the 931A type (1P121), placed immediately behind the rotating chopper. The photomultiplier is magnetically and electrostatically shielded with Netic shielding material, the light being admitted through a narrow slot directly in front of the photocathode. Both the main and reference beams are in juxtaposition directly behind the chopper, and then illuminate the photomultiplier photocathode.

The amplification circuit for the photomultiplier is patterned after a design of Applied Physics Corporation and is used with their permission. This amplifier has many desirable characteristics, among which is a feed-back circuit which maintains the current

of the 10th dynode at a constant value. In this way, the sensitivity of the phototube is invariant and phototube damage due to accidental exposure to high levels of illumination is obviated. The output of this highly stable amplifier is used to drive a sensitive detector of construction similar to that used with the lead sulphide cell.

Although a photomultiplier tube shows preferential sensitivity for certain angles of polarization, the construction of the instrument using a Rochon prism minimizes errors from this source.

When the Rochon is oriented for use as an analyzer, the emerging beam exits through that part of the crystal which is cut as a basal section. That is to say, the beam is essentially parallel to the optic axis of the crystal and the exit surface of the crystal is perpendicular to its optic axis. Since the beam is not collimated, various parts of the beam pass through the basal section at slight angles to the optic axis which, in effect, produces a partial depolarization of the exit beam. If the light falling on the photocell is examined, it is found to consist of a bright spot which contains circular black rings

and isogyres. It is also found that this pattern cannot be extinguished by means of another polarizer placed between the phototube and the Rochon prism, thus indicating that the exit beam is not linearly polarized.

Controls have been added, both to the electronic circuit and to the optical system to provide for accurate phasing of the important signal components which include: (a) residual 60 cycle hum; (b) sample beam; and (c) reference beam.

Since the amplifier is sharply peaked at 60 cps, any residual noise generated in the electronic circuitry will reach the output as a 60 cycle signal. Those components which are not cyclic in nature (such as pick-up from transformers) will contribute only small output amplitudes. However, although the magnitude of the hum signal is small, it is several times in excess of the required "sensitivity" of the instrument.

Fortunately, it is a characteristic of the phase-sensitive detector, when operated at the same frequency as the residual hum, that the latter can be effectively neutralized by adjusting phase "in quadrature".

This involves setting the detector to pass equal amplitudes of the positive and negative portions of the wave. A phase control of this kind has been added to the Polarometer amplifier so that the phase of the phase-sensitive detector can be varied to place the residual hum accurately in quadrature; the output meter, therefore, now reads zero under conditions of steady-state hum.

After balancing out steady state hum, there has been observed a fluctuating noise signal which causes the output and null balance meter to give a small erratic reading. This noise has not been completely identified but is believed to be generated by the photoelectric cell. Since cell noise is of a random nature, only 60 cycle components are passed by the amplifier. And, since these components can have any magnitude and phase, they cannot be placed in quadrature.

The sample-beam signal is phased in respect to the phase-sensitive detector by rotating the motor to which the chopper wheel is attached. This motor has been remounted for easier rotation and a

handle attached for convenience in adjusting phase.

The sample-beam phase is adjusted by rotating the plane mirror shown, in the photograph of Fig. 4, just behind the circular, neutral, density wedge. This mirror has been mounted on a rotating platform and its orientation can be adjusted through a hole in the top of the cover.

In order to provide a means of adjusting instrument range for a given set of experiments, a range control has been added to the reference beam. This is in the form of a rotating Polaroid HN22 polarizer, also seen in the photograph, and located just beyond the short tube which contains the reference-beam lens. A similar fixed polarizer, attached to the end of the tube, completes the optical arrangement and provides a wide range of adjustment, made by means of a knob on the top of the cover.

Suitable light baffles were added to the instrument after the photograph was taken.

The measuring characteristics of the neutral density wedge were investigated by blocking the

sample beam and plotting the voltage output of the amplifier (ahead of the phase-sensitive detector) as a function of rotation of the neutral density wedge. It was found that the system was linear to the limit of accuracy of the measurement and had a dynamic range of nearly two decades. The slope of the plotted line indicates the calibration of the output reading with respect to the logarithmic wedge. The light intensity measured by the instrument (in terms of scale reading) is found by multiplying the scale reading by the factor 2.175, treating the scale reading as a number between 0 and 1. The antilog of the product is then proportional to light intensity in the reference beam which is equal to light intensity in the sample-beam during a null measurement.

Greater convenience in reading the scale has been achieved by replacing the large knob and counter which had a 100 to 1 ratio with a 10-turn potentiometer dial with corresponding changes in gearing. This change was dictated by the finding that settings could be made more rapidly and with as good accuracy using a dial with lower resolution.

Measurements taken with the instrument, as modified, show promising results when tested on a series of samples having different concentrations of fluorescent material. Under these conditions, the specific fluorescence (defined as the sum of the horizontal and vertical intensity components divided by the concentration) was found to be properly constant.

III. PLANNING
October 1 to December 31, 1962

Since progress on the diphtheria system has been most encouraging, it is planned to push this phase of the investigation to completion as rapidly as possible. Immediate plans call for preparation of sufficient rhodamine-labelled toxoid to permit a variety of chemical and immunochemical measurements to be made and to thus standardize working material.

With standard material in hand, the next step will be to determine conditions for obtaining improved sensitivity using the fluorescence polarization method. In addition, procedures will be worked out for determining the quantity and avidity of antibody in a particular unknown specimen. It is expected that these will be represented, respectively, by the binding capacity for toxoid, on the one hand, and the chemical association constant, on the other. Probably both of these quantities will prove to be of practical use in determining the state of immunity of a donor.

Work on the detection of antibodies to bacterial cell walls will progress in the direction of

antibody determination by complement fixation tests, leading possibly to preparation of sera which will also give good agglutination. Even if agglutinating sera are not obtained, the inhibition studies will be carried on using the complement fixation test. In this way, the reactivity of lysozyme-degraded cell walls or papain-digested walls will be investigated. Once this has been worked out, the preparation of reagents for fluorescence polarization applications should follow in a straightforward fashion.

Instrumentation improvements will include investigation of anomalous light apparently entering the photocell when the polarizer is set for horizontal measurements, and suspected of being responsible for the negative polarization values currently obtained. Whereas negative polarization is proper for certain wave lengths of exciting light, it is not expected for the fluorescent material under study at the exciting wave length being used. This phenomenon will be examined and indicated corrective measures taken.

Steps are also being taken to obtain an easier interpretation of the readout, either by using a

linear neutral density wedg or by the construction of a logarithmic read-out dial. In either case, the necessity of multiplying by a facto- and taking antilogs will be eliminated.

Following completion of the above-described tasks, a program of continuing investigation will presumably be initiated, as outlined in a follow-on proposal to the Office of the Surgeon General.

IV.

APPENDICES

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